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**Ecdysone secretion by prothoracic glands of *Manduca sexta* in vitro.**

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PROTHORACIC GLANDS OF MANDUCA SEXTA  
IN VITRO.

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Ph.D. of the University of Bath, 1983.

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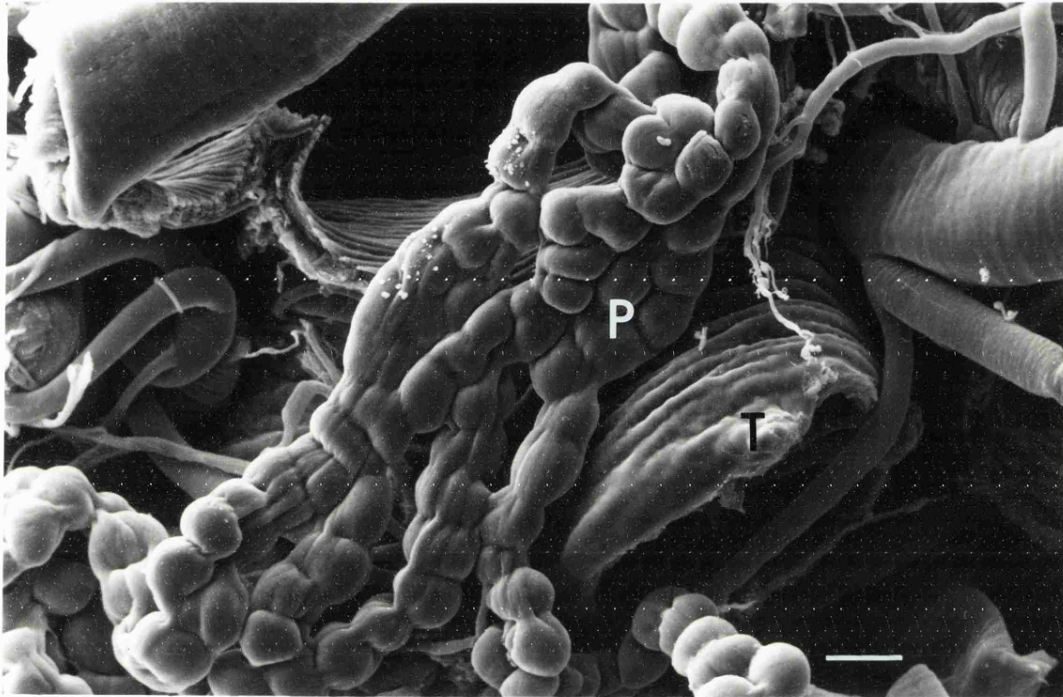
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Frontispiece: Scanning electron micrograph of prothoracic gland cells of a fifth instar Manduca sexta larva. P; prothoracic gland, T; trachea. Scale bar represents 100 microns. Photograph courtesy of N. Platt.

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## SUMMARY

Insect moulting is regulated by the steroid hormone, 20-hydroxyecdysone (20-HE), the immediate precursor of which, ecdysone, is secreted by the prothoracic glands (PG) in Lepidopterous larvae. The PG are influenced by a trophic hormone, which originates in the neurosecretory cells in the insects' brain and is known as prothoracicotropic hormone (PTTH). A third hormone, juvenile hormone (JH), directs which type of cuticle (larval, pupal or adult) is produced at each moult.

Prothoracic glands of the tobacco hornworm, Manduca sexta (Sphingidae; Lepidoptera), were maintained in short term tissue culture, and secreted ecdysone measured by radioimmunoassay. The in vitro secretory rate was calculated for glands isolated from animals at daily intervals during the last larval instar, and compared with whole animal ecdysteroid titre and with total protein content of the gland cells.

The effect of a number of humoral factors on ecdysone secretion was assessed. Whilst ecdysone itself had no effect, 20-HE was found to inhibit secretion when used at physiological levels, thus

forming the basis of a possible feedback mechanism regulating PG activity.

The responsiveness of PG to PTH activity in extracts of Manduca larval brains was investigated at various times during the last larval instar. It was found that the PG would respond to these extracts only at times when PTH is thought to be released in vivo.

The effect of JH on PG from both normal and head-ligated larvae was investigated. No influence on secretory activity of PG could be demonstrated, even at JH levels well above the in vivo titre.

The results are discussed in relation to control of in vivo PG activity, and control of whole animal titre of 20-HE.

### ABBREVIATIONS

AF1:	activation factor 1
AF2:	activation factor 2
AMP:	adenosine monophosphate
AZT:	arbitrary "zeitgeber" time
BSA:	bovine serum albumin
BST:	British Summer time
CA:	corpora allata
CC:	corpora cardiaca
CNS:	central nervous system
cpm:	counts per minute
DNA:	deoxyribose nucleic acid
EG:	wandering fifth instar larva
GLC:	gas liquid chromatography
H:	Horn antiserum
HMG:	high mobility group
HPLC:	high performance liquid chromatography
JH:	juvenile hormone
JHE:	juvenile hormone esterase
LSC:	lateral neurosecretory cell
L5:	feeding fifth instar larva
mRNA:	messenger RNA
ODC:	ornithine decarboxylase
op:	osmotic pressure

PG: prothoracic gland(s)  
PTTH: prothoracicotropic hormone  
PTU: phenolthiourea  
RIA: radioimmunoassay  
RNA: ribose nucleic acid  
SAS: saturated ammonium sulphate  
TLC: thin layer chromatography  
uv: ultra violet  
W1: Whitehead antiserum 1  
W2: Whitehead antiserum 2  
20-HE: 20-hydroxyecdysone

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## CHAPTER 1

### INTRODUCTION

Ample evidence has accumulated to show that both the temporal sequence and the qualitative expression of the insect moult cycle are under endocrine control (see Wigglesworth 1954, 1970 and Richards, 1981 for reviews). The sequence of changes which occur in the insect integument during this process is described in detail by Locke (1969a) and a brief outline will suffice to illustrate the known functions of the endocrine control system.

During the postembryonic growth and development of insects, periodic moulting occurs to allow enlargement of the confining exoskeleton, known as the integument or cuticle. Under optimal growth conditions, the number of moults is defined, dependent on the species. The moulting process imposes a superficial rhythmic punctuation on the underlying continuum of development, and is hormonally controlled.

The first endocrine component in the temporal control of each moult is the release of

prothoracicotropic hormone (PTTH) from a neurohaemal organ associated with the brain, where this hormone is synthesized. Using the haemolymph as a vector, PTTH reaches and acts on a gland in the thoracic region, causing the synthesis and release of the steroid ecdysone. The nature and hence name of this gland varies in different insects (Herman, 1967), but the term prothoracic gland (PG) has been most popular in recent literature.

The product of the prothoracic gland was shown to be important in controlling the moulting of insects by the now classical work of Wigglesworth (1934) and Fukuda (1944). Subsequent purification and characterisation revealed the moulting hormone to be the steroid ecdysone (Karlson et al, 1965; Huber and Hoppe, 1965). This compound and its 20-hydroxylated derivative have since been shown to control the moulting process in a wide variety of insects. Most of the evidence has been acquired using techniques involving manipulations of the whole animal, transplantation of endocrine organs or target tissues, administration of exogenous hormones or exposure of tissues to hormones in vitro (see

Gilbert and King, 1973; Morgan and Poole, 1976 for reviews). Recently an ecdysone deficient mutant of Drosophila has enabled demonstration that ecdysone is required for larval growth and development by non-invasive techniques (Garen et al, 1977).

Ecdysone is generally thought to be a precursor of the active moulting hormone, 20-hydroxyecdysone (20-HE), to which it is metabolised by a variety of tissues. However, the ratio of ecdysone to 20-HE may be a factor in controlling the moulting process (see below).

At the epidermis, the moulting hormone acts at the transcriptional level and is capable of producing both qualitative and quantitative changes in protein synthesis (Riddiford, 1980c). The overt effect of this is that the epidermis separates from the cuticle, a process termed apolysis, and undergoes mitosis. This often results in infolding of the detached epidermis due to increase in area after cell division. The changes in morphology of epidermal cells during the moulting cycle are documented by Locke (1969a). The epidermal cells then secrete the upper layers of a new cuticle, the morphology and

protein composition of which is directed by a class of hormones known as the juvenile hormones (JHs). These are secreted by paired organs in the head known as the corpora allata (CA) and act on the epidermal cells to direct transcription initiated by 20-HE.

The epidermis secretes a mixture of enzymes between the two cuticles, digesting the inner layers of the old one, the components of which are resorbed. Finally, the insect undergoes ecdysis, a frequently complex process under separate control, resulting in the casting off of the remnants of the old cuticle. Often, the new cuticle is significantly modified after ecdysis and does not harden in its final shape for several hours (see Reynolds, 1980 for review).

Earlier work on the physiology and control of insect moulting has been described by Wigglesworth, 1954). The endocrine control mechanisms have been reviewed several times Wigglesworth, 1964; Doane, 1972; Wyatt, 1972; Gilbert and King, 1973; Gilbert et al, 1980a; Richards, 1981) whilst the biochemistry of insect hormones is the subject of numerous discussions (Karlson, 1956a; Karlson and Seckeris, 1964;

Gilbert, 1967; Gilbert et al, 1977; Sridhara et al, 1978; Riddiford and Truman, 1978; Gilbert et al, 1980b). I will try to confine this discussion to areas most relevant to the work described.

#### PROTHORACICOTROPHIC HORMONE

The first evidence for endocrine control of insect moulting was obtained in 1917 when Kopeč demonstrated that it was possible to prevent the posterior half of a Lymantria dispar larva from moulting by isolating it from the anterior portion using a ligature, applied 10 days prior to moulting. This type of experiment, which has since contributed a great deal to our knowlege of the endocrinology of insect development, along with nerve sectioning techniques, demonstrated that the involvement of the head in moulting was humorally mediated (Kopeč, 1922). Thus, PTTH was in fact the first insect developmental hormone to be discovered, and our relative ignorance of its chemical nature and mode of action seems particularly ironic.

The necessity for a factor from the brain to initiate moulting was confirmed by a variety of workers, using a number of different insects (Fraenkel, 1935; Kühn and Piepho, 1936; Bodenstein, 1938; Bounhiol, 1938; Schmeider, 1942; Williams, 1952a, b). Wigglesworth (1934, 1940), by use of decapitation and reimplantation experiments, demonstrated that a factor released into the blood from the head of Rhodnius initiated moulting. He dissected various parts of the brain and nervous system and implanted them into decapitated larvae to demonstrate that the hormone occurred in groups of neurosecretory cells (the lateral neurosecretory cells or LSC) identified by Hanström (1938). Subsequent work involving microscopic examination of these cells in Locusta gave additional evidence for their involvement in the control of the moulting cycle (Highnam and West, 1971). Modern techniques have subsequently allowed the identification of a single neurosecretory cell in each LSC group of Manduca as being the origin of PTH (Agui et al, 1979). However, the exact nature of the PTH producers may vary in different insects (Riddiford, 1980d).

### Development in the absence of PTTH.

Despite the accumulation of evidence to suggest PTTH to be responsible for control of the onset of moulting, there are numerous reports of moulting and development in insects deprived of their supposed source of PTTH, the brain (Ishizaki, 1972; Malá et al, 1977; Meola and Adkisson, 1977). Perhaps the most relevant to the present study is the report by Judy (1972) that debrained Manduca larvae could undergo moulting, pupation and adult development after brain extirpation. The time scale of these processes was much delayed relative to the normal temporal sequence. A careful study of the role of the brain in initiation of developmental processes in Manduca (Safrenek and Williams, 1980) showed brain activity was essential for normal development and that other ganglia were incapable of substituting for the brain. Also, implants of brains from diapausing or non-diapausing animals to brainless recipients resulted in development conforming to the temporal pattern anticipated in the donors. Hence, the temporal control of the development of Manduca is determined by the

brain, via its timed release of PTH. That moulting and development can occur in debrained animals was not questioned. The point is that moulting in the debrained animals should be seen as the result of abnormal processes initiated in the absence of the temporal regulator residing in the brain.

Sehnal et al (1981) found Galleria PG eventually became activated (after a delay of 30 to 40 days) in animals deprived of their brains. They concluded, as the characteristics of the rise in ecdysone titre were the same as in normal, PTH stimulated animals, that PTH normally acts as a trigger, the subsequent control of ecdysone titre being independant of PTH. JH was also shown to influence the release of PTH by transplanted brains, as well as modifying the response of the PG to PTH and itself being capable of indirectly activating pupal PG in situ. The role of PTH in activating the PG is therefore much more complex than was initially supposed. However, it remains safe to say that in normal development PTH plays a vital role in the temporal control of ecdysone release.



### Neurohaemal release site for PTH

It was assumed for a long time, on the basis of anatomical and histological investigations, that the release site for PTH was the corpora cardiaca (CC) (Gilbert and King, 1973). This assumption was based on the above mentioned evidence for the LSC being the origin of PTH, and the observation that in insects deprived of their cerebral neurosecretory cells, the CC ceased to accumulate neurosecretory material (Highnam, 1962). However, this reasoning has been questioned by the recent demonstration that in Manduca sexta the CA appear to be the release site for PTH, as they contain five times the PTH activity of the CC (Agui et al, 1980). However, both the CC and the CA are capable of release of PTH in vitro (Carrow et al, 1981). The demonstration that cobalt backfills of neurones which project from the brain of Manduca to the CC stain the cell bodies thought to be responsible for PTH production has compounded this question (Buys and Gibbs, 1981). The CC remain likely candidates for the release of a variety of other products of the

cerebral neurosecretory cells (Highnam and Hill, 1969).

In the case of the slug moth, Monema flavescens, it has been suggested that neurosecretory material is released directly from neurosecretory cells in the pars intercebralis to the haemolymph, where it is taken up by haemocytes (Takeda, 1976b, see below). This observation may explain the numerous reports that isolated brains when implanted into insects and so deprived of their connections to the CC and CA, are able to release PTTH in an apparently normal manner.

#### Control of PTTH release.

The release of PTTH precipitates a moult. What then precipitates the release of PTTH? This problem has been investigated in Rhodnius, where abdominal stretch receptors, activated after the animal has gorged itself with a blood meal, send nervous impulses to the brain, stimulating PTTH release (Wigglesworth, 1934; 1964). More recently, increased electrical activity of neurosecretory cells in Rhodnius brains have been correlated

with periods of PTH release (Orchard and Steele, 1979).

There is also evidence suggesting that sensory input to the brain is important in allowing PTH release. In Galleria mellonella, close confinement of final instar larvae which were capable of pupation delayed this process for at least 10 days (Edwards, 1966; Sehnal and Edwards, 1969). Similar results were obtained by Alexander (1970) who suggested nervous control of the PG as the likely explanation, although Woolever and Pipa (1970; Pipa, 1970) favoured neuronal control of PTH release, comparable to that observed in Rhodnius, to explain similar results. In a more recent report, Malá and Sehnal (1978) found that whilst sectioning the nerve cord of Galleria larvae between the metathoracic and first abdominal ganglia would delay pupation, additional sectioning between the brain and suboesophageal ganglion abolished this effect. As implanting an active brain to these operated animals still caused normal development, they concluded that this was evidence for neuronal control of PTH release, though the method of

control must be complex. The role of the nervous supply to the PG is discussed further below.

PPTH release has also been shown to be influenced by several environmental stimuli, including photoperiod (Truman, 1971) and temperature (Williams and Adkisson, 1964). Of special interest is the finding that in some species, the region of the brain which is responsible for PPTH synthesis is photosensitive, and so regulation of PPTH production by photoperiod is mediated within the brain itself (Williams and Adkisson, 1964; Truman, 1976; Steele, 1976). There is evidence to suggest the activation of PPTH synthesis to be dependant on cyclic AMP (Rasenik et al, 1976). The role of extraretinal photoreception in controlling hormonally regulated aspects of insect physiology has been reviewed by Truman (1976). (For a discussion of other factors influencing brain neurosecretory activity, see Gilbert and King, 1973, Gilbert et al, 1980b).

Humoral control of PPTH release has been suggested for a number of species of insect. Steele (1973, 1975) has provided histological evidence for ecdysteroids regulating the synthesis

of PTH in Rhodnius, thus effecting a feedback control over ecdysone production. In Mamestra brassicae, both ecdysone and JH have been implicated in the control of PTH synthesis and release. Agui and Hiruma (1977) presented histological evidence that ecdysone released by the PG acts to suppress release, but increase synthesis of neurosecretory material in the type 2 neurosecretory cells of the pars intercerebralis. Conversely, a lack of ecdysone (produced by extirpating the PG) induced release of this material. Similar techniques used by Hiruma et al (1978b) gave results implying JH may regulate PTH release at certain times during the normal development of Mamestra.

In the blowfly, Calliphora vicina, both JH and 20-HE have been shown to affect the synthesis of RNA and protein in the brain (Scheller and Bodenstein, 1981). The effects of both compounds varies with developmental stages used, the cessation of feeding and onset of wandering (see below) being correlated with a change of response. Whilst it is uncertain that these results are in any way linked with PTH synthesis or secretion, they do indicate the

possibility of humoral modulation of brain activity.

In a variety of studies on the regulation of insect diapause, JH has been implicated as a controlling factor, presumably acting by exerting control over PTH release. In these studies, PTH has been assumed to be responsible for the termination of diapause, by causing a release of ecdysone. A high JH titre is associated with maintenance of larval diapause in a number of insects and so it has been postulated that this inhibits the release of PTH (Chippendale and Yin, 1973; Yagi and Fukaya, 1974; Chippendale, 1977; Takeda, 1978; Yin and Chippendale, 1979). In other species, JH has been postulated to be involved in diapause induction and not needed for maintenance. Presumably, the PTH producing cells once induced to enter a diapause state either await a second signal to terminate diapause, or rely on an endogenous timing mechanism (Sieber and Benz, 1977; Chippendale and Yin, 1979).

In the case of the termination of pupal diapause by PTH release, temperature and photoperiod have been shown to be important

regulators in a number of insects (Williams, 1969; DeWilde, 1970).

In the insect under investigation in this project, Manduca sexta, growth is of primary importance in the control of moulting. The ability to release PTH in the fifth larval instar appears to be related to the weight attained by the animal (Nijhout and Williams, 1974a). This control is apparently mediated by the level of JH in the haemolymph. When a critical weight (around 5g) is attained, JH is eliminated from the haemolymph, and the brain becomes competent to release PTH (Nijhout and Williams, 1974a, b).

Elimination of JH from the haemolymph is ensured by the temporally precise appearance of a specific juvenile hormone esterase (JHE, see below; Vince and Gilbert, 1977). A similar correlation between JHE activity and ecdysone titres also occurs in Galleria mellonella (Hwang-Hsu et al, 1979), indicating that a comparable control system for PTH may exist in a number of insect species. In starved larvae, the drop in haemolymph JH levels is not achieved, and moulting to a supernumerary larval stage occurs (Jones et al, 1980). This indicates that whilst

decline in JH levels correlates with PTH release and may precipitate it, PTH release will occur in the presence of JH in the fifth instar, as it does during previous instars. Evidence from Trichoplausia (Jones et al, 1981) indicates haemolymph levels of JHE can be affected by brain extracts. Hence, it is likely that the changes in JHE, JH and ecdysone levels which bring about the metamorphic moult are all controlled by the brain with, perhaps, feedback regulation of JH to coordinate the temporal sequence of events.

In Manduca, actual release of PTH occurs during a precise period in the daily light cycle, termed a photoperiodic gate (Truman and Riddiford, 1974). It should be noted that this growth dependent mechanism controlling PTH release has been demonstrated for the larval/pupal moult only, although release of PTH in earlier instars is also subject to photoperiodic gating. Photoperiodic gating of PTH release dependent on a circadian rhythm has been found to occur in other insects, including Samia cynthia ricini (Fujishita and Ishizaki, 1981).

A recent study of the problem of the influence of JH on PTH release in Mamestra led



Sehna et al (1981) to conclude that JH acts to modify release of PTTH from transplanted brains, as well as acting at many other points in the regulation of ecdysteroid titre. However, at no time did JH directly influence the secretion of ecdysone by the PG, as has been suggested in some systems (see Chapter 7).

#### Assay techniques for PTTH

Determination of the chemical nature of this hormone has proved anything but simple, partly due to difficulties encountered in its assay. Methods of assay have until recently relied upon whole animal preparations deprived of their own source of PTTH, but in a suitable state for the PG to show the normal response<sup>to</sup> the hormone, ie. ecdysone secretion and subsequent development. Thus PTTH is measured indirectly by observing overt changes due to a rise in ecdysone titre (Gersch, 1961; Kobayashi and Yamazaki, 1966; Gibbs and Riddiford, 1977). Of these methods, that used by Gibbs and Riddiford utilising a neck ligated preparation of a fourth instar larva of Manduca sexta has been the most sensitive and enabled the

demonstration of PTH activity in a single group of neurosecretory cells from the brain of a Manduca pupa.

Kambysellis and Williams (1971) pointed the way to a more sensitive and reliable PTH assay by demonstrating the in vitro activation of PG from diapausing Cynthia silkworm pupae. Successful activation was demonstrated by spermatogenesis in co-cultured testes. Agui (1975) performed similar experiments with PG from diapausing Mamestra brassicae pupae. Brains from non-diapausing pupae co-cultured with PG from pupae 40 days post pupation gave successful activation. The secreted ecdysone was measured by an in vitro assay utilising Chilo suppressalis integument (Agui et al, 1972; Agui, 1973). Obviously, these types of PTH assay systems were extremely time consuming, and the relative insensitivity of the techniques used for quantifying the secreted product of the PG (ecdysone) detracted from the advantages of an in vitro system.

The application of ecdysone radioimmunoassay (RIA, see below) to this problem resulted in the most rapid and sensitive PTH assay yet designed

(Bollenbacher et al, 1979). This assay utilises incubated PG from freshly pupated Manduca sexta. Secreted ecdysone is measured by RIA on a crude methanolic extract of the culture medium (Bollenbacher et al, 1975). The assay is extremely sensitive, detecting as little as 0.1 brain equivalents, compared to the limit of 0.4 brain equivalent detected by the whole animal technique devised by Gibbs and Riddiford. This comparison of sensitivities is inexact, however, as the former report refers to larval brains whilst the latter refers to non-diapausing pupal ones.

#### The chemical nature of PTTH

PTTH has proved extraordinarily time consuming to characterise and purify, essentially due to difficulties encountered in its measurement. An early attempt at characterisation using debrained pupae as an assay system led to the isolation of an ether soluble substance with PTTH activity, implying that the hormone was a lipid (Kobayashi and Kirimura, 1958). After extraction and purification from  $10^6$  Bombyx

mori brains, Kobayashi et al (1962a, b) isolated a substance with high PTH activity which was identical to cholesterol. As cholesterol is a major constituent of insect tissues at all stages of development, this was unlikely to be the true identity of the brain hormone. Work by Carlisle and Ellis (1963) showed that whilst an impure preparation of cholesterol accelerated moulting in late fourth instars of Locusta, a purified preparation had no activity.

Other workers claimed that the brain hormone contained a proteinic substance (Ichikawa and Ishizaki, 1961, 1963), and further experiments (Kobayashi and Yamazaki, 1966) suggested both sterol and protein constituents of the brain extract to have PTH activity. Yamazaki and Kobayashi (1969) suggested PTH to be a glycoprotein of about 20Kd. Some of this early data was reviewed by Williams (1967) who added to the debate by speculating that PTH was a mucopolysaccharide.

More recent work, using a variety of different bioassays, has supported the idea that PTH is a peptide (and hence stable to some proteases) with a molecular weight of around 5Kd

(Gersch et al, 1977; Ishizaki et al, 1977; Nasagawa et al, 1979; 1980). In a preliminary report, Kingan and Newburg (1979) suggest that PTH from wandering fifth instar Manduca is a protein of 45Kd molecular weight. The recent introduction of a rapid and sensitive in vitro assay for PTH is expected to speed progress in elucidating the chemical structure of this hormone (Bollenbacher et al, 1979). Preliminary results have suggested that PTH may exist in two forms in Manduca pupal brains (Gilbert et al, 1981).

#### Mode of action of PTH

Work on the mode of action of PTH at the PG has been hampered by the lack of a pure preparation of the hormone. However, it has been possible to correlate changes in PG physiology with developmental stage and thus PTH activation. Oberlander et al (1965) correlated synthesis of nucleic acids in PG with their stage of development in two silkworm species, PG activity correlated with increased RNA but not DNA levels. Gersch's group have provided evidence for two

factors present in the CC of Periplaneta which act on the PG. The first of these, activation factor 1 (AF1) stimulates RNA synthesis, but not ecdysone production by the PG (Brauer et al, 1976). AF2 has been shown to increase the membrane potential of the PG, thus possibly affecting the permeability of the glands (Gersch et al, 1973). It appears that the PG of Periplaneta are influenced by two hormonal stimuli during the last instar. The first, probably AF1, causes a rise in RNA content, which resembles the documented pleiotypic effect of a number of hormones (see Shields, 1978). This may prepare the synthetic apparatus of the gland cells for steroid synthesis. The second stimulus causes a more specific response, resulting in ecdysone synthesis and secretion (Gersch, 1980). It is not known how AF2 fits into this scheme of PG activation.

Experiments on cultured Manduca PG suggest cyclic AMP plays a role in the activation of ecdysone secretion (Vedekis and Gilbert, 1974; Vedekis et al, 1974; 1976). PTTH may, therefore, cause increased levels of adenyl cyclase activity, producing cyclic AMP as an intracellular second

messenger in the manner of numerous vertebrate hormones.

### ECDYSTEROIDS

The term ecdysteroids is used to describe the family of steroids structurally related to ecdysone (Goodwin et al, 1978), which was the first of these compounds to be identified (Butenandt and Karlson, 1954). Whilst seven identified ecdysteroids have been isolated from insects (Gilbert et al, 1980a), the two most important in regulating the Manduca moulting cycle are ecdysone and 20-HE (Fig 1.1, see above). Besides their action in post-embryonic insect development, ecdysteroids are also present during insect oogenesis (Legay et al, 1976; Dorn and Romer, 1976; Dinan and Rees, 1981) and embryogenesis (Kaplanis et al, 1975; 1976; Hoffman et al, 1980) and are synthesised by the ovaries of some insects (Lageux et al, 1977; Goltzené et al, 1978; Hagedorn et al, 1975; Ohnishi et al, 1977). These ecdysteroids may have roles in both oocyte development (Legay et

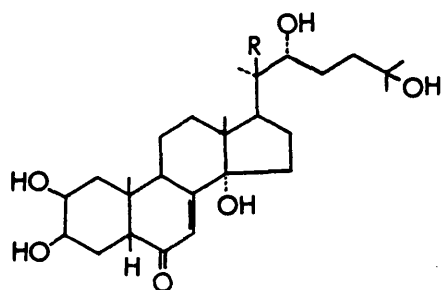


FIG. 1.1: STRUCTURAL FORMULA FOR ECDYSONE ( $R = H$ )  
AND 20-HYDROXYECDYSONE ( $R = OH$ ).

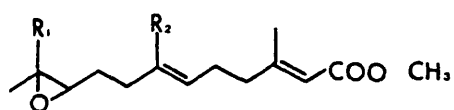


FIG. 1.3: STRUCTURAL FORMULA FOR THE JUVENILE HORMONES.  
JH1;  $R_1 = R_2 = C_2H_5$ , JH2;  $R_1 = C_2H_5$ ,  $R_2 = CH_3$ , JH3;  
 $R_1 = R_2 = CH_3$



al, 1976; Bownes, 1982) and embryogenesis and the prehatching embryonic moults of insects (Lageux et al, 1979; Hoffman et al, 1980), and may represent metabolism of maternal components rather than ecdysteroid synthesis by the embryo (Garen et al, 1977; Hanaoka and Ohnishi, 1974). The biosynthetic pathways involved in ecdysteroid production are reviewed by Rees et al (1980).

#### Timing of ecdysone secretion.

Until quite recently, ligation experiments (see above) remained the major technique for determining times of action of insect hormones (see Chapter 3). In Manduca sexta, the temporal variation in ecdysteroid titre has been measured accurately by biochemical techniques (Wielgus et al, 1979; Riddiford, 1980a, 1981, Fig. 1.2). A similar pattern of ecdysone levels has been reported in a number of insects, with a large peak of ecdysteroids characterising both metamorphic (larval-pupal or pupal-adult) and non-metamorphic (larval-larval) moults. An earlier, much smaller peak has been detected prior to the metamorphic moult of several holometabolous

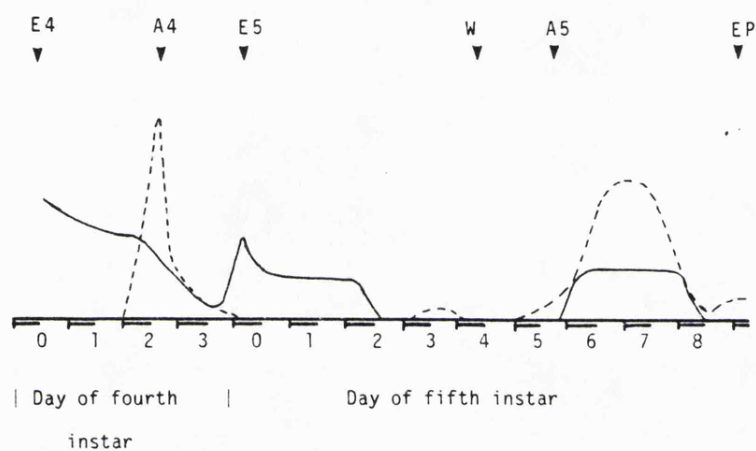


FIG. 1.2: LEVELS OF ECDYSTEROIDS (---) AND JUVENILE HORMONE (—) DURING FOURTH AND FIFTH INSTARS OF *MANDUCA* (ARBITRARY UNITS). E4; FOURTH INSTAR ECDYSIS, A4; FOURTH INSTAR APOLYSIS, E5; FIFTH INSTAR ECDYSIS, W; ENTRY TO WANDERING STAGE, A5; FIFTH INSTAR APOLYSIS, EP; PUPAL ECDYSIS. MODIFIED FROM RIDDIFORD (1981).

species including Manduca (Bollenbacher et al, 1975), Leptinotarsa (Hsiao et al, 1976), Philosamia (Calvez et al, 1976), Pieris (Lafont et al, 1975; 1977), Galleria (Hsiao and Hsiao, 1977; Hwang-Hsu et al, 1979) Tenebrio (Delbeque et al, 1978a), and Calpodes (Dean et al, 1980).

The regulation of this titre is obviously precise and, as the amount of circulating hormone is dependent upon the discrepancy between rates of synthesis and degradation of hormone, could be effected by control over either or both of these parameters. As it seems wasteful of energy for a compound to be synthesised to excess, the organism relying on subsequent degradation to prevent undesirably high levels accumulating, we may expect regulation of synthesis to be a major controlling factor. Indeed, correlations of in vitro PG ecdysone secretion and whole animal titre led both Bollenbacher et al (1975, working on Manduca) and Hirn et al (1979, working on Locusta) to conclude PG activity to be the major regulator of whole animal ecdysteroid levels. However, before investigating how control of production may act to regulate the ecdysteroid

titre, it is essential to ascertain which tissue(s) are active in its production.

#### Site of ecdysone synthesis

The now classic experiments of Fukuda (1944) demonstrated that the PG were vital to the induction of pupation. More recent reports of production of ecdysone by PG in vitro (see below) have proven the PG to be ecdysone secreting tissues. Further evidence is provided by the immunocytochemical demonstration of ecdysteroids in Galleria PG (Birkenbeil et al, 1979) and observation of signs of precursor uptake and steroid secretion by this tissue (Blazek and Malá, 1978).

Ecdysone production from tissues other than the PG has been reported (Bonner-Weir, 1970; Locke, 1969b; Nakanishi et al, 1972; Romer et al, 1974; Bulliere and Bulliere, 1974; Hsiao et al, 1975; Studinger and Willig, 1975; Gersch and Eisbich, 1977; Delbeque et al, 1978b; Delbeque and Sláma, 1980), as has continued moulting of insects deprived of their PG (Chadwick, 1955; Nutting, 1955). It is likely that in some insects

the production of ecdysteroids for pupal development is accomplished by tissues other than the PG, although these function to produce ecdysteroids for larval moulting (Glitho et al, 1979; Delbeque and Slama, 1980). Likely candidates for this role are the oenocytes (Locke, 1969b; Romer et al, 1974), based on ultrastructural and histological similarities with steroidogenic tissues.

However, King et al (1974) concluded that in Manduca at least, the PG were the major if not the only tissue synthesising ecdysone in late fifth instar larvae. Experiments described here (see Chapter 4) show that the amount of ecdysone produced by the prothoracic glands may be adequate to account for the known whole animal titre.

Insects are unable to synthesise the steroid nucleus (Clayton, 1964; Gilbert, 1967; see Thomson et al, 1973 for review) and therefore are dependant on a dietary supply of sterols, often cholesterol. (Earlier reports that aphids were an exception and able, either intrinsically or via their symbionts, to synthesise sterols have recently been cast doubt upon by careful experiments by Campbell and Nes (1983), which

revealed that sterol requirements were met by the diet, and sterol biosynthetic intermediates were not detectable in the animals.) Whilst it is unlikely to be a factor regulating ecdysone production in vivo, possible substrate limitation has been cited as a reason for suboptimal ecdysone secretion by PG in vitro (Bollenbacher et al, 1975). The biosynthesis of ecdysteroids is reviewed in detail by Gilbert et al (1977) and Rees et al (1980).

#### Control of ecdysone synthesis

Factors which may influence ecdysone secretory rates are numerous. Whilst PTH has been shown to be the major trophic hormone to stimulate the PG (Gilbert et al, 1977; Bollenbacher et al, 1979) we remain in ignorance of how the glands are switched off. Possible effects of ecdysteroids and JH on the secretion of ecdysone by the PG are discussed separately (see below).

It has long been known that the PG of a number of insects are well innervated (Lee, 1948; Williams, 1948; Scharrer, 1964; Beaulaton, 1968b; Hintz-Podufal, 1970; Blazek et al, 1975;

Granger, 1978; Benedeczky et al, 1980) and the PG of Manduca are no exception (Chapter 2). Some of these nerves appear to have neurosecretory droplets in their endings (Srivastava and Singh, 1968; Hintz-Podufal, 1970; Blazek et al, 1975; Benedeczky et al, 1980) and connect the glands to the prothoracic and suboesophageal ganglia and the inter-ganglionic connectives. Granger (1978) demonstrated the cell bodies of neurones supplying the PG of Galleria lay in the suboesophageal, prothoracic and mesothoracic ganglia, but was unable to determine the type of neurone involved. The function of these nervous connections remains in doubt. While Possompes (1953) concluded that they were essential to the normal functioning of the glands, Srivastava et al (1977) found no effect of sectioning the nerves supplying the PG in Papilio larvae (see Granger 1978 for discussion).

Whilst in Manduca, the CA appear to be involved in storing PTH (Agui et al, 1980), both the CA and CC have been shown to be capable of releasing it (Carrow et al, 1981) and PTH has been measured in haemolymph (Gilbert et al, 1981), there remains the possibility that the

haemolymph is not the only, or even the major vector for PTH. The neurosecretory nerve endings observed may indeed represent an as yet uninvestigated supply route to the PG for PTH. Alternatively, these nerves may be involved in gland maturation, or in the inhibition of gland activity. Carlisle and Ellis (1968) suggest the lateral neurosecretory cells of the locust protocerebrum may produce a PG inhibiting substance, though its supply route to the PG was uninvestigated.

Evidence has been found to suggest that in some insects the haemocytes play a role in controlling the activity of the PG. In Monema flavescens they appear to function in transporting neurosecretory material from the B cells of the pars intercebralis to the PG (Takeda, 1977). Whilst Wigglesworth (1955) showed haemocytes to be indispensable to the normal moulting of Rhodnius and suggested a role for them in PTH activation of the PG, or in ecdysone production, he subsequently concluded that this role was not PTH transport (Wigglesworth, 1956). The role of the haemocytes in insect moulting still remains obscure. Hoffman (1972) reported



that x-irradiation of haemopoietic tissue of Locusta early in the last larval instar results in prolonged delay of moulting. However, the PG appear to become active as normal, and are able to induce moulting when transplanted to last larval instars whose PG have been extirpated (Joly et al, 1973; Hoffman and Weins, 1974). It may be that haemocytes function in 20-hydroxylation of ecdysone or have a role at the epidermis.

There is evidence to suggest that the PG of some insects are temperature sensitive. Browning (1981) found the ability of the PG of Heliothis punctiger to respond to PTH depended on the temperature at which they underwent pharate pupal development. Whilst experiments suggested this effect was directed on the PG, it is possible that other temperature receptors modify the secretory ability of the glands by nervous or other communication.

#### Regulation of ecdysteroid titre

Whilst synthesis of ecdysone is the sole factor likely to increase the whole animal titre, reductions in the level of ecdysteroids may be

effected by three mechanisms, degradation, excretion and sequestration. The physiological contribution of each of these mechanisms is unclear. Degradation of ecdysteroids can be by conjugation to produce sulphate and glycoside esters (Heinrich and Hoffmeister, 1970; Yang and Wilkinson, 1972), oxidation to both 3-dehydro (Koolman and Karlson, 1975, 1978) and 26-hydroxy (Svoboda et al, 1975) derivatives (although these latter have some biological activity) and isomerisation to less active forms (Nigg et al, 1974). Excretion of both ecdysone and 20-HE, as well as their 3-dehydro derivatives has been described (Morgan and Poole, 1977).

The significance of ecdysone sequestration is uncertain, although a specific haemolymph binding protein for 20-HE has been described which may render the hormone inactive and therefore affect the ecdysteroid titre available to the tissues (Emmerich, 1970a, b; Feyereisen 1977, 1980), despite earlier reports of no ecdysone carriers in the haemolymph of Hyalophora (Chino et al, 1970; Gorell et al, 1972; Gilbert and Chino, 1974). The detection of ecdysone glycosides in incubations of Calliphora brain and ring gland

complexes led Willig et al (1971) to postulate a role for such conjugates in ecdysone transport or storage.

Excretion of ecdysteroids and their metabolic products has been documented in a number of insects (Morgan and Poole, 1977). In Locusta, excretion of ecdysone, 20-HE and their 3-dehydro derivatives has been demonstrated, and occurs in a stage specific manner (Hoffman and Koolman, 1974). However, the role of excretion in regulation of ecdysteroid titre remains unclear (see Chapter 8 for further discussion). A full account of ecdysteroid metabolism and degradation is given in two recent reviews (Riddiford and Truman, 1978; Gilbert et al, 1980b).

#### Which is the true moulting hormone?

During the synthesis of the mammalian oestrogens, a number of hormonally active precursors are formed including pregnenolone and progesterone. Indeed, the role of the large quantities of androgens produced by the fetal testis is confusing until it is realised that most is used in oestrogen synthesis by the endocrine

placenta (Petropoulos, 1982). The analogy in insects is the secretion of ecdysone by the PG (Chino et al, 1974; Borst and Engleman, 1974; King et al, 1974; Bollenbacher et al, 1976) which is converted by a variety of tissues (but not the PG) to 20-HE by C-20 hydroxylation (King and Siddall, 1969; Moriyama et al, 1970; King, 1972; King and Marks, 1974; Hoffman et al, 1975; Bollenbacher et al, 1975; Nigg et al, 1976). An exception to these observations is the in vitro synthesis of both ecdysone and 20-HE by the PG of Monema flavescens, reported by Takeda (1976a). 20-HE is the more biologically active of the two forms in a variety of assays (Ashburner, 1971; Chihara et al, 1972; Milner and Sang, 1974; Oberlander, 1974; Fain and Riddiford, 1977; Mandaron et al, 1977; Riddiford, 1978;) and has been found to be present at sufficiently high levels to account for ecdysteroid induced events in vivo (Borst et al, 1974). In contrast, Panitz et al (1972) report ecdysone more active than 20-HE in the activation of Balbiani rings on salivary gland chromosomes of Acrictopus lucidus and Clever et al (1973) found some puffs on Chironomus

salivary gland chromosomes more sensitive to ecdysone than 20-HE, whilst for others the order was reversed. Oberlander found only ecdysone could prolong DNA production by Galleria wing disks in vitro and 20-HE was inhibitory to DNA synthesis. He proposed that ecdysone initiates metamorphosis while the rising levels of 20-HE subsequently turn off DNA synthesis while allowing continued morphogenesis (Oberlander, 1969, 1972). Whilst the ratio of ecdysone to 20-HE varies during development (Bollenbacher et.al, 1975) this perhaps should be viewed as a reflection of the changing expression of ecdysone 20-hydroxylase (Nigg et al, 1976) rather than evidence for differential roles of the different ratios (Riddiford, 1978).

#### Mode of action of Ecdysteroids

Pioneering work on ecdysteroids (Clever and Karlson, 1960) allowed Karlson (1963) to formulate a theory for the action of steroid hormones which has become the basis for current understanding. Steroid hormones are known to pass through cell membranes and bind to specific cytoplasmic

receptor proteins. The hormone-receptor complex then passes to the nucleus, where it binds to specific regions of chromatin and modifies the expression of the genome (O'Malley and Schrader, 1976). This change in gene expression is mediated by changes in RNA synthesis, which are translated by the cell into a change in protein populations. This concept of steroid hormone action has been documented in numerous different systems (Schulster et al, 1976).

Most of the evidence supporting the hypothesis that ecdysteroids, in particular 20-HE, act in the same manner as other steroid hormones has been produced by work on insect cell lines and imaginal discs in vitro. Imaginal discs are groups of cells found in larvae of holometabolous insects which do not differentiate until metamorphosis, when they become the adult specific structures of the mature insect or imago. This development is stimulated by 20-HE (Postlethwait and Schneiderman, 1970) and the system is amenable to in vitro study.

20-HE, in common with other steroid hormones, is known to be bound by cytoplasmic receptors, and the complex to accumulate in the nucleus (O'Malley

and Schreider, 1976; Maroy et al, 1978; Yund et al, 1978; Schaltmann and Pongs, 1982). Ecdysone is known to cause changes in gene expression at the target cells (Berendes, 1967) and it has been shown that 20-HE generates RNA synthesis from specific puff sites on polytene chromosomes of Drosophila (Bonner and Pardue, 1977). 20-HE has been located at these ecdysteroid inducible puff sites (Gronnemeyer and Pongs, 1980) and is also known to bind to the chromatin in Manduca epidermal cells (Dyer and Riddiford, 1980). Recently, Savakis et al (1980) have demonstrated induction of specific polypeptides by ecdysteroids in a Drosophila cell line. Similar findings from work on Manduca epidermal cells in vitro are discussed below.

The hypothesis of Kroeger (1966) that ecdysone exerts its effect in altering gene activities, visualised as chromosome puffs, by modulating the ionic balance of cells has been a subject of some debate (Ashburner and Cherbas, 1976; Kroeger, 1977) and has received little attention in recent publications. The mode of action of ecdysteroids is generally agreed to be similar to that of other steroid hormones. The

action of 20-HE at the target tissues is discussed below.

### THE JUVENILE HORMONES

JH was first demonstrated by Wigglesworth (1934) as a blood borne factor which inhibits metamorphosis in Rhodnius. There are at least three different species (JH1, JH2 and JH3, Fig. 1.3), named in order of discovery. JH1 was identified by Röllner et al (1967) and JH2 by Meyer et al (1968), both from the cecropia silkworm. JH3 was isolated from Manduca five years later (Judy et al, 1973). The chemistry and biology of the different JHs is reviewed by Dahm et al (1976) and their occurrence and functions have been summarised in a number of reviews (Gilbert and King, 1973; Gilbert et al, 1980a; Riddiford, 1980a, b).

JH is synthesised and secreted by the CA (Judy et al, 1973; Granger et al, 1979), at precisely controlled intervals during the insect life cycle. Determinations of titres during



post-embryonic development shows temporal and quantitative fluctuations, which require accurate control mechanisms (Bergot et al, 1976; Fain and Riddiford, 1975; Hsiao and Hsiao, 1977).

The mechanisms of control of titre are not known, but possibilities include control of synthesis, catabolism and excretion. There exist JH specific binding proteins which render JH both inactive at the target tissue, and unavailable to esterases (Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973; Kramer et al, 1974; Sanburg et al, 1975; Gilbert et al, 1978). In Locusta at least, JH binding proteins exist which are capable of discriminating between the different JHs, perhaps allowing selective control of titres (Peter et al, 1979). These processes are reviewed in detail by Gilbert et al (1976, 1977, 1980b) and DeKort and Granger (1981). Of particular interest is the temporally precise appearance of a JH esterase in Manduca (Weirich et al, 1973; Vince and Gilbert, 1977) which is specific for the hormone-carrier complex (Sanburg et al, 1975) at a time when it is of vital importance for normal development that the JH

titre is low (Nijhout and Williams, 1974b; Riddiford, 1980a, see below).

In Manduca, JH has been measured and correlated with levels of ecdysteroids at various times during the larval-pupal transformation (Fain and Riddiford, 1975; Riddiford, 1981). The temporal relationship of fluctuations in the titres of these two developmental hormones has become the basis for present understanding of the way in which 20-HE and JH act together to regulate sequential expression of larval, pupal and adult phenotypes.

Besides its role in controlling the pattern of gene expression during metamorphosis, JH functions in a number of other aspects of insect physiology. These include regulation of larval diapause (Chippendale, 1977), various aspects of insect behaviour (Truman and Riddiford, 1977) and vitellogenesis (Hagedorn and Kunkel, 1979).

#### Role of JH and ecdysteroids in epidermal metamorphosis.

The classical action of JH is to prevent precocious development of insects. That is, it is

present at high levels at larval moults to maintain expression of the larval characters, low levels at pupal moults (in holometabola) to allow expression of pupal characters and absent at adult moults, permitting formation of the mature insect.

Wigglesworth (1940) considered that this action was achieved by JH specifically activating the expression of larval characters. Thus, if JH is administered during the adult moult, larval characters are reexpressed, resulting in a "juvenilised" adult, and hence the pupa may be regarded as a larval-adult intermediate. The phrase "status quo" was coined by Williams (1952a) to describe the action of JH. This more accurately represents the presently accepted role of JH, that is to prevent alteration of the pattern of genomic expression when 20-HE initiates synthesis of a new cuticle prior to moulting. Under this hypothesis, as formulated by Williams and Kafatos (1971), the larval, pupal and adult stages are seen as the result of expression of three different gene sets and thus the pupa represents expression of a unique set of characters. In fairness to Wigglesworth it must be pointed out that the "status quo" hypothesis is formulated with

specific reference to holometabolous insects such as Lepidoptera, whilst his original hypothesis referred to homometabola such as Hemiptera, in which a true pupal stage is absent.

The detailed investigation of this hypothesis has been pursued furthest in the study of the expression of cuticular proteins by the epidermis of Manduca (see Riddiford, 1980c for review). At a larval moult, the epidermis is exposed to a surge of ecdysteroids in the presence of JH (Fig. 1.2). This response can be mimicked in vitro and, if JH is absent, a pupal moult results (Riddiford et al, 1980). The same precocious pupal moult can be elicited in vivo (Fain and Riddiford, 1976). Prior to the pupal moult, two ecdysteroid peaks are observed (Fig. 1.2; Bollenbacher et al, 1981). The first peak is small, but very important. It represents the first occasion in the life of the insect on which the epidermis is exposed to ecdysteroids in the absence of JH. This results in a transient apolysis (Riddiford and Curtis, 1978) and an irreversible commitment of the epidermis to pupal gene expression (Nijhout, 1976; Riddiford, 1976a; 1978) and can also be mimicked in vitro. A

role for ecdysone in changing the commitment of, or reprogramming the genome has also been suggested by work on Calliphora where the first peak of ecdysteroid produces a quantitative and qualitative change in RNA synthesis in epidermal cells (Shaaya, 1976).

Change of commitment coincident with exposure of the epidermis to 20-HE in the absence of JH has been documented in two other Lepidopterans, Galleria (Hwang-Hsu et al, 1979) and Bombyx (Calvez, 1980). However, in both these insects, pupal cuticle secretion can be stimulated in some individuals by 20-HE injections during the feeding stage of the last larval instar. It may be that the ability of the epidermis to secrete pupal cuticle is acquired prior to the first surge of ecdysteroids in these animals, the "reprogramming" peak serving only to prevent further larval gene expression. Alternatively, as JH titres were not measured in these animals and high doses of 20-HE were used (30µg/g in Galleria), it is possible that the injections themselves caused cellular reprogramming and subsequent cuticle deposition. In Galleria at least, it was demonstrated that JHE activity had reached high levels in the

haemolymph prior to the first time at which pupal cuticle deposition could be stimulated.

During the second ecdysone surge JH is once more present and may protect the adult-specific genome from premature expression for, whilst it is not needed under normal circumstances for a pupal moult (Mitsui and Riddiford, 1976; 1978), JH does appear necessary to protect some imaginal structures from precocious adult development (Kiguchi and Riddiford, 1978). Such precocious adult development can be prompted in some systems by continuous low levels of ecdysteroids in the absence of JH (Nardi and Willis, 1979; Riddiford, 1980c).

The transition to adult gene set expression appears to be achieved in a similar way. Hence, if JH is present at the onset of the rise in ecdysteroid titre, a second pupal cuticle is formed (Williams, 1961; Riddiford, 1980c).

This view of the role of JH in regulation the independant expression of the three cuticle types under ecdysteroid stimulation, leads to the conclusion that there are three different gene sets, any one of which may be exclusively

expressed by an epidermal cell, depending on the hormonal milieu it experiences.

The molecular basis for the action of JH and ecdysteroids.

An analysis of the molecular events occurring over the period prior to and during the pupal moult of Manduca is presented by Riddiford (1981).

DNA synthesis occurs at the time of the first ecdysone release (Wielgus et al, 1979), but appears to be neither ecdysone dependant nor necessary for commitment to pupal gene expression (Wielgus et al, 1979; Dyer et al, 1981). DNA synthesis has also been found <sup>not</sup> necessary for change of commitment of epidermal cells of Galleria (Krishnakumaran, 1978). In contrast, mRNA and protein synthesis during exposure to 20-HE in the absence of JH are vital to cellular reprogramming (Riddiford et al, 1981). 20-HE has been shown to produce an increase in RNA production in a variety of systems (Shaaya 1976; Siegel and Fristrom, 1978; Sridhara et al, 1978). However, analysis of the RNA produced during the change of

commitment showed qualitative changes in the mRNA populations (Chen and Riddiford, 1981; Riddiford and Kiely, 1981). Essentially, the change at commitment represents a loss of synthesis of a large number of mRNA species coding for larval cuticular proteins, with a few new mRNAs appearing. These new mRNAs code for proteins not typical of either pupal or larval cuticle and may be a part of the intracellular commitment mechanism.

Three mRNA species coding for pupal type proteins are produced about 36 hours after exposure to the commitment surge of ecdysone, with the bulk of this type of RNA appearing about 24 hours before pupal ecdysis, ie. about 72 hours later. Synthesis of protein follows these changes in mRNA populations. Interestingly, the pupal commitment mRNAs do appear to be translated in vivo (Riddiford and Kiely, 1981). If JH is present during the in vitro change in commitment, then the larval type cuticular protein mRNAs continue to be synthesised, and the pupal commitment mRNAs do not appear.

The exact way in which JH modifies the action of 20-HE on the epidermal cells remains unclear.



Like 20-HE, JH also has cytoplasmic receptors (Klages et al, 1980; Chang et al, 1980) which accumulate in the nucleus in the presence of the hormone (Riddiford and Mitsui, 1978), although chromatin binding has not been demonstrated. Riddiford (1981) suggests JH may act by preventing the 20-HE receptor complex from binding to the chromatin, perhaps by steric hindrance from the JH receptor complex bound to an adjacent site.

Willis (1981) has suggested a role for JH in inhibiting the known role of 20-HE in induction of ornithine decarboxylase (ODC). ODC is known to be vital to the production of polyamines in a variety of animal tissues. Polyamines have been shown to be active in allowing cell phenotype to be altered, possibly by releasing high mobility group (HMG) proteins, which are thought to function in controlling the activity of regions of DNA (Weissbrod and Weintraub, 1979; Weissbrod et al, 1980), from chromatin. However, so far there is no evidence to suggest that polyamines are active in the 20-HE dependent reprogramming of insect cells, and whilst JH3 does act to block ODC induction in bovine lymphocytes (Kensler et al, 1978), a specific inhibitor of ODC (a-difluoromethyl

ornithine) failed to mimic the action of JH in Oncopeltus (Willis, 1981). However, as Willis points out, the similarities between the action of 20-HE in the reprogramming of Manduca epidermal cells and the action of polyamines in the reprogramming of cells of a variety of systems, make this hypothesis worthy of further investigation.

#### Rationale for present study.

From the foregoing discussion, it is apparent that the fluctuating titre of ecdysteroids is a major factor controlling insect development. Control of the titre may be effected by a number of means (see above), but rate of secretion of ecdysteroids is likely to be of great importance. As the intact insect represents a complex system with respect to influences on ecdysteroid levels, it is necessary to be able to study a part of this system in isolation to gain an understanding of its interaction with the multitude of variables acting on it in vivo. The part of the ecdysteroid titre regulating system under study

here is the ecdysone producer, the prothoracic gland.

This project is an attempt to analyse the responses of the gland to a number of physiologically relevant blood borne factors. Isolation of the gland from the normal, complex in vivo environment is achieved by in vitro techniques. The tobacco hornworm, Manduca sexta, was chosen as the best available experimental animal primarily due to the accumulated body of knowledge concerning endocrine aspects of its development (see Chapter 2).

## CHAPTER 2

### STUDIES ON THE PROTHORACIC GLANDS OF MANDUCA SEXTA.

Much of what is known about endocrine aspects of postembryonic insect development has been discovered by work on holometabola, especially the Lepidoptera (see Chapter 1). Holometabolous insects exhibit three dramatically different appearances during development, ie. larval, pupal and adult stages. The transitions between these (the superficial manifestations of which are the metamorphic moults) represent unique developmental periods which can be compared and contrasted with each other and with non-metamorphic moults to obtain information about the factors controlling development.

The North American Sphingid, Manduca sexta (the Tobacco Hornworm) is a relative newcomer to the range of Lepidoptera utilised for this type of investigation. During earlier work, silkmooths (Saturniidae) were the preferred species because of their large size and ready availability due to commercial interest. However, more recent

work on Manduca has revealed features of its endocrinology which render it particularly suitable for study (Truman, 1972; Truman and Riddiford, 1974).

The terminology used in this project to describe periods during insect development is that advocated by Hinton (1968). The term instar defines the period between two successive apolyses, while stage is more loosely applied to refer to a period of the insect life cycle during which the animal has a characteristic external appearance. It should be noted that ecdysis, therefore, often occurs early in the instar and hence pupal ecdysis, for example, refers to the casting off of the fifth larval cuticle by the newly formed pharate pupa. The wandering stage thus embraces the end of the fifth and beginning of the pupal instars (see below). The term moult refers to the complete process of development from one instar to the next, and encompasses the physiological preparation for apolysis, apolysis itself and subsequent events leading up to ecdysis.

Under optimal growth conditions, Manduca larvae develop from newly hatched first instar

larvae to pupae in about 19 days. Upon hatching from the egg, the first instar larva commences feeding and, as it grows, moults successively to four more larval instars. The final larval instar (the fifth) differs in its biology from any of the previous four. The first to fourth instars grow, stop feeding and moult to the next larval instar. The fifth instar feeds and grows for about four days, then ceases feeding but delays moulting for a further four to five days. During this time it exhibits a characteristic change in appearance and behaviour and is referred to as the wandering stage.

On entering the wandering stage, fifth instar larvae lose the pigment (insecticyanin, Cherbas, 1973) from the epidermis overlying the dorsal blood vessel, which thus becomes visible. There are less dramatic pigment changes all over the epidermis, rendering the animal a different hue from younger fifth instars. During the wandering stage animals are initially very active (for the first 2 days), exhibit characteristic burrowing behaviour, and attempt to chew through any obstacles. At this time the gut is emptied and so when Manduca are kept in captivity, the onset of

wandering is easily recognised by an overnight transformation in the insect's living quarters, caused by these activities of the larva.

Wandering behaviour ceases after about two days, and apolysis to the pharate pupal stage occurs. Pupal ecdysis follows after about three more days. Depending on photoperiod, pupae either immediately initiate adult development, which culminates in ecdysis of the mature insect (eclosion) in 18 to 19 days, or enter a pupal diapause, which causes initiation of adult development to be delayed for several months. A light regimen of 17 hours light ; 7 hours dark (17L;7D) allows continuous development, whereas 12L;12D induces pupal diapause.

#### Timing of developmental events

Under diapause inducing conditions, it has been demonstrated that there exists in Manduca a circadian rhythm which limits the release of PTH to a narrow temporal period each day (Truman, 1972). This permissive period is termed a photoperiodic gate, and the phenomenon is referred to as "gating" (Pittendrigh and Skopik, 1970). The

result of this time-dependent release of PTH, and hence ecdysone, is that animals become developmentally synchronised at each PTH release. Hence, careful study of the moult to the fifth larval stage, using neck ligatures applied at different times to developmentally synchronous animals, demonstrated that the earliest time at which head ligation will no longer prevent all animals from moulting was 1.5 hours prior to lights out, whilst the latest time that head ligation could prevent any animals from moulting was 8.5 hours after lights out. This type of study cannot define exactly the periods of hormone secretion, but it does indicate times when subsequent development becomes independent of the endocrine gland concerned, and thus gives an indirect indication of its periods of activity. Thus, the gate allowing PTH secretion in fourth instar Manduca is open for 10 hours, from 1.5 hours prior to lights out until 8.5 hours after lights out. When dealing with photoperiodically entrained rhythms, it is conventional to designate times as arbitrary "zeitgeber" times (Pittendrigh, 1965) or AZT. Thus, as lights out is designated midnight (24.00), the fourth instar PTH release



gate is open from 22.30 until 8.30 AZT (Truman, 1972).

Not all Manduca larvae grow at exactly the same rate. PTH release in the fifth instar is dependent upon growth (Nijhout and Williams, 1974a) and this is almost certainly true of earlier instars. Hence, for a population of animals of similar ages, some become competent to release PTH before others. However, the gating phenomenon described imposes a developmentally synchronising effect on this variable growth rate. Fourth instar animals which become competent to release PTH during the period when the gate is closed must wait until 22.30 AZT to initiate PTH release. Synchrony is not absolute, however, as animals becoming competent between 22.30 and 8.30 AZT are able to release PTH immediately. This results in animals which are developmentally synchronous at fourth instar ecdysis becoming split into two groups during preparation for fifth instar ecdysis. Some faster growing animals become competent to release PTH on the second night after fourth instar ecdysis, during the time the PTH gate is open. Others become competent after the gate closes, and so must wait until 22.30 AZT

the following evening to release PTTH. These two groups are designated gate 1 and gate 2 animals (Truman, 1972).

Close investigation of the larval to pupal moult of Manduca revealed a similar system (Truman and Riddiford, 1974; Fain and Riddiford, 1976). Due to the gated release of PTTH during the fourth instar, developmentally synchronous pharate fifth instar larvae can be obtained. Of such a group, about 50% enter the wandering stage during the fourth night after ecdysis, the remainder wait until the fifth night. That no animals enter the wandering stage during the day implies that the PTTH release controlling this phenomenon is also gated and so larvae become resynchronised at this time. Unlike the gate for PTTH release prior to fourth instar apolysis, the pre-wandering PTTH gate occurs during the photophase, and PTTH secretion lasts from 17.00 AZT to 20.30 AZT. These two synchronising events allow the comparison of different individual fifth instar larvae to be made with a high degree of confidence that they will be at similar developmental stages.

The developmental synchrony of Manduca larvae, their large size and rapid growth, make it

a very useful experimental animal and a number of laboratories have turned their attention to studying its biology. Hence, there are now available measurements of the levels of ecdysone and JH during the larval-pupal transition and also the beginnings of an understanding of how these fluctuating hormone titres regulate the dramatic events at the epidermis (see Riddiford, 1981, for review and references).

#### Maintenance of experimental animals

Manduca sexta larvae were maintained at 26°C in a high humidity atmosphere (about 45% relative humidity) in two rooms. One was maintained in a 12L:12D (short day) photoperiod to provide experimental animals, the other in a 17L:7D (long day) photoperiod to provide non-diapausing pupae for egg production. Lights off was at 5.00 p.m. BST in the short day room, and at 10.00 p.m. BST in the long day room. Larvae were fed on an artificial diet which was a modification of that specified by Bell and Joachim (1976). All materials used were reagent grade unless otherwise specified.

To prepare the diet, 20g of agar ("Lab m" agar No. 2; London Analytical and Bacteriological Media Ltd. Salford.) were mixed with 500mls of tap water and autoclaved for 45 minutes. 168g of pre-mixed solid ingredients (Table 2.1) were added to 350mls of boiling water and mixed in a blender. The agar solution was added plus 2mls of corn oil (Mazola), 2mls of raw linseed oil (Polycell) and 4mls of 10% formaldehyde (BDH Chemicals Ltd. London). The mixture was blended to uniformity, and allowed to cool to 70°C. Finally, the following antibiotics and vitamins were added: 0.1g Vanderzant vitamin mixture (ICN Nutritional Biochemicals Ltd. supplied by Uniscience Ltd. Cambridge, England); 2g aureomycin (Lederle Laboratories, division of Cyanamid of Great Britain Ltd. Gosport, Hants.); 4g ascorbic acid (Sigma London Chemical Co. Ltd. Poole, Dorset, England.), and the diet placed in trays to set. Diet was stored at 4°C for up to 4 days, or at -20°C for longer periods.

Eggs were collected at daily intervals and allowed to hatch in small (about 10ml) plastic pots. Hatching took place in 2 to 3 days, and newly hatched first instar larvae were placed on

TABLE 2.1: Solid ingredients for Manduca diet.

Ingredient	Weight (g)	Supplier
Casein	350	BDH
Wheatgerm (Beemax)	750	Grocery <sup>1</sup>
Sucrose	300	Grocery <sup>1</sup>
Dried yeast	150	DCL <sup>2</sup>
Wessons salts	100	Uniscience: ICN
Sorbic acid	15	Sigma
Cholesterol	10	Sigma
Methyl-4-hydrocybenzoate	10	Sigma
Choline chloride	10	Sigma

These ingredients were mixed and stored refrigerated prior to use. See text for details of Manduca diet preparation. <sup>1</sup> Grocery indicates product intended for human consumption obtained from a local retail shop. <sup>2</sup> DCL; Distillers Company (Yeast) Ltd. Sutton, Surrey. See text for addresses of other suppliers.

approximately  $1\text{cm}^3$  pieces of diet in small pots. Animals were maintained in these conditions, with diet changes every 3 to 4 days until apolysis to pharate fifth instar larvae.

To obtain experimental animals, fourth instar larvae showing head capsule slippage (ie. apolysis) and newly ecdysed fifth instars were collected daily during the period between 16.00 and 18.00 AZT and placed in large (about 200mls) plastic pots with an approximately  $2.5\text{cms}^3$  piece of diet. The diet was changed after 2 days and, after wandering, animals were placed in a clean plastic pot with a paper tissue to absorb moisture lost by the animal due to the emptying of the gut, which continues for several hours. The larvae completed pupal development in this cup, and pupae ecdysed five days after wandering. Once the pupal cuticle had hardened, the animals were placed in wooden trays which were checked daily for pharate adults. These latter were recognised by darkening and thinning of the cuticle. They are readily apparent upon handling, as the cuticle is easily deformed. Such animals were used for breeding, along with those reared in the long day room.

Animals were treated in the same manner in the long day room, except that on wandering they were placed in 2.5cm diameter holes in unseasoned wooden (elm) blocks and the holes corked. Pupae were collected about 10 days later and placed in a wooden box to eclose and lay eggs. This adult cage was maintained in long day lighting conditions, with a dim (4W) lamp switched on constantly, in a high humidity atmosphere (>45%). It measured about 1M<sup>3</sup>, to allow the animals to fly, as mating and oviposition generally occurs during flight. A wire mesh fence was provided to allow newly eclosed adults to hang freely and expand their wings, although many were capable of climbing the plywood sides of the box. The air in the box was filtered constantly to remove scales shed by the moths, as these cause considerable irritation to workers. Adults were allowed to feed from a 25% sucrose solution in a container beneath a hollow yellow plastic flower. A cut tobacco leaf, or an intact plant, was provided and females laid eggs singly at the periphery of the leaves, from where they were collected by hand. Females can mate and lay eggs on the first night after eclosion, and lay up to around 500 eggs/night for 2 to 3 nights.

### Growth rate of fifth instar *Manduca* larvae

Short day animals maintained under the conditions described grew at a similar rate to that described by Truman and Riddiford (1974) and Nijhout and Williams (1974a; see Table 2.2). Larvae were synchronised at ecdysis to the fifth instar by selection of pharate and newly ecdysed fourth instars as described.

Animals were timed from the lights out preceeding this ecdysis. Hence, newly selected fourth instars and pharate fifth instars at, for example, 10am BST would be 17 hours post the lights off preceding their fifth instar ecdysis (lights out was 5.00pm on the previous day, see above). The age of these animals is abbreviated to L5;0.17, indicating they are zero days and 17 hours after the beginning of the day on which they underwent fifth instar ecdysis. Three days later, animals are 3 days and 17 hours post fifth instar ecdysis and are termed L5;3.17.

Approximately 50% of these animals will undergo wandering on the following night (Table 2.2). Weighings of large numbers of L5;3.17 larvae showed that very rarely did an animal weighing



TABLE 2.2: Weights and entry to wandering stage of  
Manduca larvae of various ages.

Age	Weight (g)	N	Number entering wandering stage
L5;1.17	2.93 +/- 0.14	26	0
L5;2.17	4.9 +/- 0.16	22	0
L5;3.17	6.78 +/- 0.21	26	14
L5;4.17	8.61 +/- 0.21	24	24
EG;0.17	7.8 +/- 0.15	28	-
EG;1.17	5.11 +/- 0.17	19	-

Weights expressed as mean +/- standard errors.

more than 6.5g at that age fail to undergo the transition to the wandering stage on the following night. However, this method of predicting wandering was not infallible, probably due to varying growth rates of individuals. The "decision to pupate" is actually made during the photoperiod of the previous day (at about L5;2.20) when the larvae weigh at least 5g (Nijhout and Williams, 1974a). Presumably, an animal which weighed too little to begin the process culminating in PTTH release (see Nijhout and Williams, 1974b), but which grew rapidly between L5;2.20 and L5;3.17 could weigh over 6.5g at L5;3.17 and still not undergo PTTH release that day, so being delayed in wandering until the night following L5;5.00.

Hence, for experiments involving gate 1 fifth instar larvae, animals weighing over 7g at L5;3.17 were selected. The remainder of the cohort (ie. animals developmentally synchronous from the fifth instar ecdysis) which had not wandered were designated gate 2 animals at L5;4.17, and invariably underwent the transition to the wandering stage the following night, though occasionally (2 out of 24, weighing 6.74g and 6.9g) they still had not attained 7g by L5;4.17.

Animals were resynchronised after wandering. Again, timing was from the lights out preceding the event and so, at 10.00am BST on the morning after wandering, both gate 1 and gate 2 animals which had wandered were collected and designated EG;0.17, indicating that these animals were 0 days and 17 hours after the beginning of the day on which they emptied their gut ("EG").

#### ANATOMY OF MANDUCA PROTHORACIC GLANDS.

Prothoracic glands vary in their anatomy in different groups of insects (Herman, 1967) but in Lepidoptera take the form of long chains of transparent cells, usually associated with the tracheal trunks originating at the prothoracic spiracle. These cells are enclosed in a sheath of extracellular material (Beaulaton, 1968a) through which nerves and tracheoles pass to the gland cells. The anatomy of the PG of a number of Lepidopteran species has been described, including Hyalophora (Herman and Gilbert, 1966), Antheraea (Beaulaton, 1968a), Galleria (Blazek

et al, 1975) and those of Manduca have a similar morphology.

A brief study was made of the anatomy of the PG during the fifth instar. Numbers of cells were counted in each of five pairs of PG from animals of various ages (Table 2.3). From these results it appears that cell number is constant during the fifth instar and so cell division does not occur.

To observe the innervation of the PG, intra vitam methylene blue staining was applied to dissected, living preparations. Using this technique, nerves became stained blue prior to other tissues and can thus be identified and observed.

### Method

A solution of 10.5% aqueous methylene blue (100ml) was mixed with 3 drops of hydrochloric acid (24%). This mixture was filtered, and 10mls warmed gently whilst stirring, avoiding boiling. Rongalite C (sodium formaldehyde sulfoxylate, 10% aqueous) was added dropwise until the solution changed from blue to a straw-yellow colour, when it was allowed to cool. Once a clear solution with a yellow precipitate had formed, the solution was

TABLE 2.3: Numbers of cells in prothoracic glands of fifth instar Manduca larvae of various ages.

Age	Numbers of cells <sup>1</sup>
L5;0.17	337.7 +/- 3.12
L5;1.17	343.9 +/- 3.25
L5;2.17	316.6 +/- 3.32
L5;3.17 (gate 1 and 2)	348.5 +/- 3
L5;4.17 (gate 2)	336.7 +/- 3.71
EG;0.17	322.3 +/- 3.05
EG;1.17	332.1 +/- 3.77

<sup>1</sup> numbers of cells given as means with standard errors of 10 prothoracic glands.

filtered and allowed to stand for at least 24 hours. This solution remains usable for 8 days, and was added directly to the tissue, under saline.

Figure 2.1 shows the anatomy of the PG and their apparent nervous supply. Points of innervation were identified by attachment of nerves to the gland sheath, so that gentle pulling on the nerve would cause an equal movement of the PG, and vice-versa. It can be seen in the figure that 8 separate points of innervation were identified. It remains possible that more points exist, but were undetected.

#### GROWTH OF PROTHORACIC GLANDS DURING THE FIFTH INSTAR.

The growth of PG was assessed by assaying the total protein content of the gland cells. As PG consist of specialised steroid secreting cells, this should give an estimation of the relative ability of the PG to synthesize and secrete ecdysone.

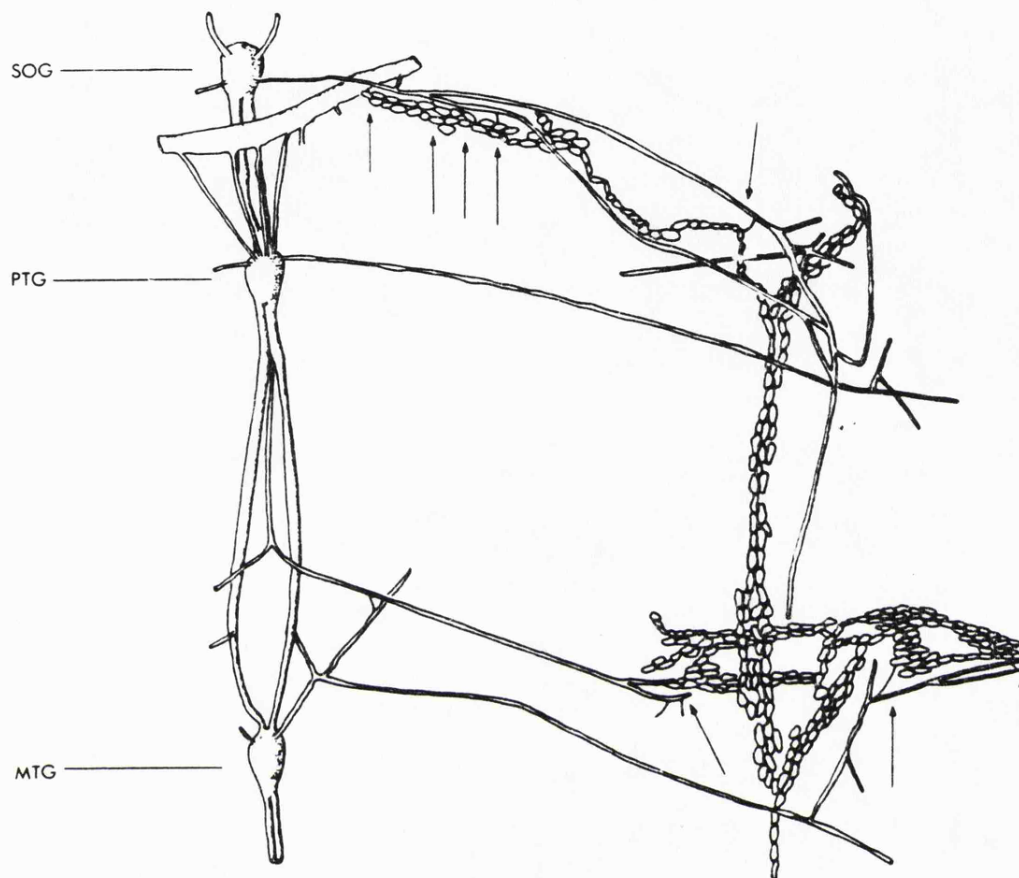


FIG 2.1: ANATOMY OF FIFTH INSTAR MANDUCA PROTHORACIC GLAND, SHOWING INNERVATION DETECTED BY INTRA VITAM METHYLENE BLUE STAINING. SOG; SUBESOPHAGEAL GANGLION, PTG; PROTHORACIC GANGLION, MTG; MESOTHORACIC GANGLION. ARROWS INDICATE POINTS OF INNERVATION.

## Methods

Glands were dissected from Manduca larvae of various ages, and carefully cleaned of non-gland material such as tracheoles and nerves. One pair of PG was sonicated in 500 $\mu$ l of distilled water, and an aliquot of 200 $\mu$ l used in the assay. All samples were assayed in duplicate. Standard solutions were made up from bovine serum albumin (BSA) in distilled water, and a range of standards from 5 $\mu$ g to 60 $\mu$ g included in each experiment.

The method for this total protein assay is modified from that described by Lowry et al (1951). Four stock solutions in distilled water were prepared as follows:

Solution A: 1M sodium hydroxide

Solution B: 0.187M sodium carbonate (2% w/v)

Solution C: 0.071M potassium sodium tartrate  
(2% w/v)

Solution D: 0.04M copper sulphate (1% w/v).

Solution C was stored at 4°C, the rest at room temperature. Immediately prior to use, a solution of 2.5mls A, 50mls C and 50mls D was prepared. 1ml of this was added to 50mls of B to prepare



solution E. Folin reagent (BDH) was diluted to 1M from a 2M stock with distilled water.

To perform the assay, 200 $\mu$ l 1M NaOH were added to the 200 $\mu$ l sample in a glass test tube. 2mls of solution E were added and the tube contents mixed immediately. After 10 minutes incubation at room temperature, 200 $\mu$ l of 1M Folin reagent were added and again the tubes were immediately mixed. Tubes were incubated for 30 minutes at room temperature prior to measurement in a Cecil 272 uv spectrophometer at 750nm. Blank values were obtained by substituting 200 $\mu$ l of distilled water for the assay sample and were subtracted from each reading. A standard curve ranging from 5 $\mu$ g to 60 $\mu$ g was constructed and results of experimental tubes calculated from the graph (Figure 2.2).

### Results

Protein content of PG was measured at daily intervals between L5;1.17 and EG;0.17 during the fifth instar (Figure 2.3). It can be seen that a dramatic increase in protein content occurs between days 2 and 3 of the instar. This increase was compared to the gain in whole animal weight by

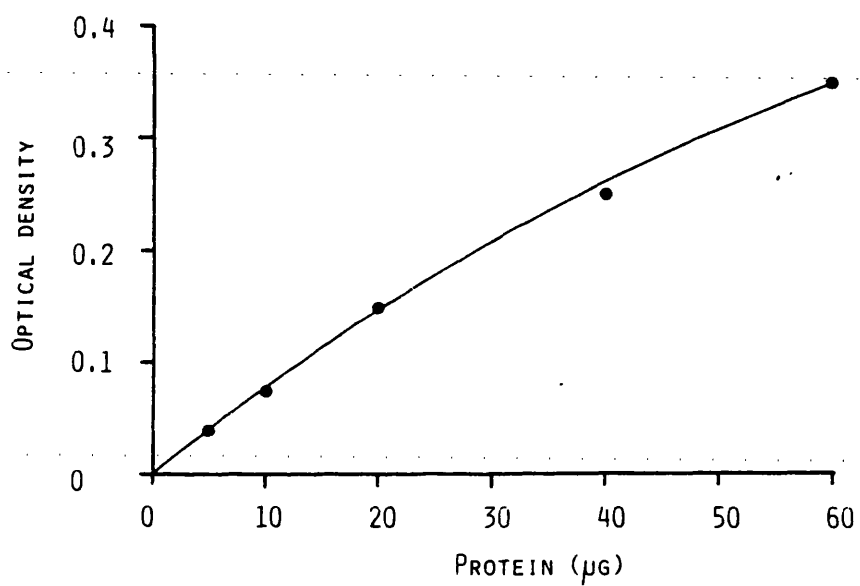


FIG 2.2: TYPICAL STANDARD CURVE FOR TOTAL PROTEIN ASSAY. POINTS REPRESENT MEANS OF DUPLICATE RESULTS. IN ALL CASES BOTH RESULTS FIT WITHIN THE SYMBOL. SEE TEXT FOR METHODS.

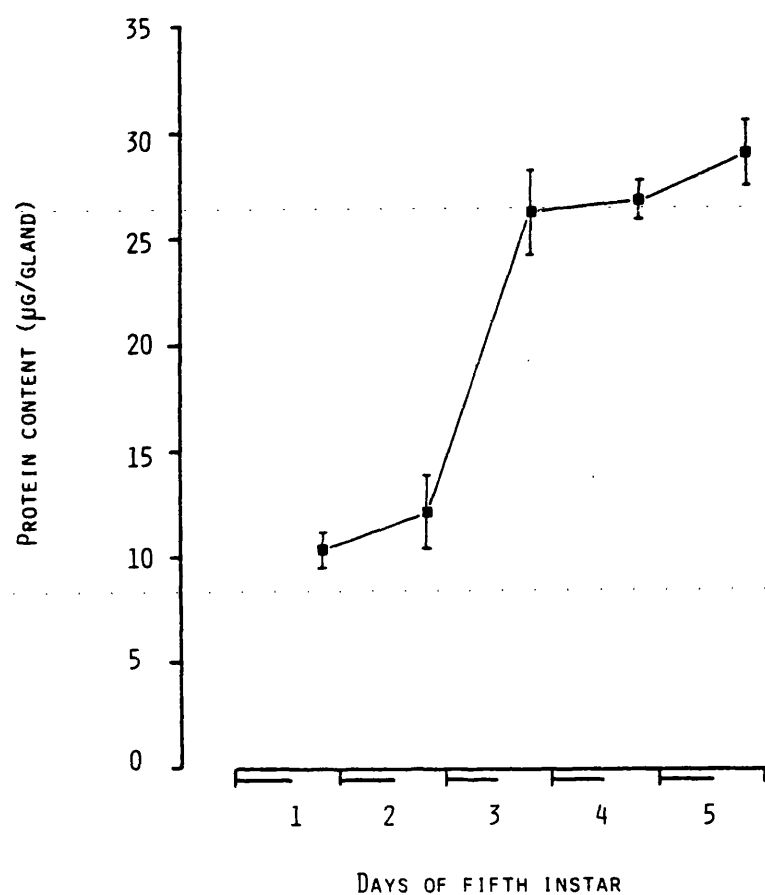


FIG. 2.3: TOTAL PROTEIN CONTENT OF PROTHORACIC GLANDS DURING THE FIFTH INSTAR. POINTS REPRESENT MEANS AND STANDARD ERRORS OF AT LEAST FIVE DETERMINATIONS. BARS ON ABSCISSA REPRESENT HOURS OF DARKNESS.

plotting the ratio of PG protein content to whole animal weight against whole animal weight (Table 2.4). This table clearly shows that during the period between day 2 and day 3, the PG increase in protein content proportionally faster than the weight gain achieved by the larva.

Examination of the individual weights of the L5;3.21 animals used in this experiment revealed that 6 out of 7 were gate 2 animals (recognised by their failure to attain the weight threshold by L5;3.17) and yet had already achieved the rapid rise in PG protein content noted above. These animals would not yet have released PTTH (Truman and Riddiford, 1974) but the decline in JH titre which precedes PTTH release, and probably facilitates it, would have begun (Nijhout and Williams, 1974b).

Hence, the rapid phase of protein synthesis observed in fifth instar Manduca PG precedes the pre-wandering release of PTTH, but is temporally correlated with the endocrinological events which are thought to prepare for it. These results are discussed further in Chapter 8.

TABLE 2.4: Whole animal weight and prothoracic gland protein content of fifth instar Manduca larvae.

Age	Whole animal weight (g)	Protein content of prothoracic gland ( $\mu$ g)	Protein content/ Whole animal weight ( $\mu$ g/g).	N
L5;0.17	2.15 +/- 0.13	7.13 +/- 0.71	3.51	5
L5;1.20	3.86 +/- 0.18	10.3 +/- 0.85	2.67	8
L5;2.20	4.73 +/- 0.28	12.08 +/- 1.75	2.55	7
L5;3.20	7.09 +/- 0.55	26.2 +/- 2.02	3.7	7
EG;0.20	6.83 +/- 0.27	26.84 +/- 0.86	3.93	5
EG;1.20	5.83 +/- 0.21	29.0 +/- 1.61	4.97	6
EG;2.20	4.8 +/- 0.05	33.23 +/- 1.67	6.92	3
EG;3.20	4.92 +/- 0.18	18.21 +/- 1.36	3.7	10
P;0.20	4.2 +/- 0.27	20.05 +/- 1.03	4.77	4

Results given as means with standard errors.

### CHAPTER 3

#### ECDYSONE RADIOIMMUNOASSAY

The definitive test for the presence of a hormone is measurement of its physiological action in a living system, that is, a bioassay. However, bioassays tend to be expensive, in terms of time and experimental animals, and are often of fairly low sensitivity when compared to chemical or immunological assays. Once the chemical species representing the active hormone is characterised, assays based on identifying and quantitating this directly can be devised. Alternatively, antisera may be raised against a hormonally active preparation, and used as a means of purifying and quantifying the hormone. Problems with this latter approach only arise when dealing with mixtures containing molecules with the same or similar antigenic determinants as the active hormone. These various approaches to hormone quantification are discussed below with specific reference to ecdysteroids.

### Selection of assay procedure

Various whole animal bioassays are available for ecdysone quantification (Kaplanis et al, 1966; Ohtaki et al , 1967; Williams, 1968; Thomson et al, 1970; Fraenkel and Zdarek, 1970). However, the techniques involved tend to be time consuming and of inadequate sensitivity (typically 0.1 to 0.5µg) to allow the determination of ecdysone titres in single organisms. In vitro assays using responses of insect tissues to ecdysone have also been devised, having better sensitivity, down to levels of 100ng (Williams and Kambyzellis, 1969; Oberlander, 1969; Mandaron, 1970; Chihara et al, 1972; Agui, 1973). This is still of inadequate sensitivity to assay ecdysone secretion by isolated PG in short term tissue incubations.

Three methods are available which are capable of detection and quantification of nanogram amounts of ecdysteroids. These are gas liquid chromatography (GLC), high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). Other techniques are significantly less sensitive (see Morgan and Poole 1976 and Morgan and Wilson

1980 for reviews, Table 3.1 for relative sensitivities.

Of the three techniques mentioned, GLC is the most sensitive and, if used coupled to an electron capture facility, as little as 5pg of ecdysteroid can be detected. However, the need for extensive sample purification, and derivatisation of the product represents a considerable drawback when large numbers of samples are to be assayed. The equipment required for optimal sensitivity is expensive, and was not available in our laboratory.

HPLC is considerably less sensitive, allowing detection of (theoretically) as little as 10ng of ecdysteroid if used with uv absorption detection (Morgan and Poole, 1976). This technique requires no modification of the sample, but as crude extracts of biological material may contain a variety of uv absorbing compounds, some sample purification may be needed. Formation of phenanthreneboronates of ecdysone, which can be separated on HPLC, has allowed detection of as little as 1ng of ecdysteroid (Poole et al, 1978).



TABLE 3.1: Relative sensitivities of various ecdysone assays.

Technique	Sensitivity (pmol.)	Reference
<u>Calliphora</u> bioassay	750	Karlson and Shaaya, 1964
<u>Musca</u> bioassay	300	Kaplanis <u>et al</u> , 1966
TLC with fluorometric detection:		
in situ	1000	Mayer and Svoboda, 1978
post elution	50	Koolman, 1980
HPLC (uv absorption detection)	2	Poole <u>et al</u> , 1978
GLC (electron capture detection)	0.01	Morgan and Poole, 1976
Mass fragmentometry	0.2	Miyazaki <u>et al</u> , 1973
RIA	0.04	Reum and Koolman, 1979

Both HPLC and GLC allow the separate quantification of ecdysone and 20-HE, an important consideration when working with whole animal or tissue extracts.

RIA has the advantage of requiring little sample purification and no modification of the chemical structures to be detected. Sensitivity is determined by the specific activity of the radioligand used and the avidity of the antibody. With suitable reagents, detection of as little as 100pg of ecdysteroid can be achieved routinely (see below). A potential disadvantage of the RIA technique is cross reactivity of different ecdysone metabolites with the antibody employed.

RIA was chosen for the quantitation of ecdysteroids in this study for ease of operation, availability of reagents and equipment, and convenience over other techniques. As will be seen, the specificity of the RIA technique was not an issue in this decision, as only ecdysone was required to be quantitated.

### RADIOIMMUNOASSAY FOR ECDYSONE

Use of RIA for the quantification of ecdysteroids was first reported by Borst and O'Connor (1972). They produced an antiserum against an oxime acetic acid ether derivative of 20-HE coupled to bovine serum albumin (BSA), by immunisation of rabbits. Using this antiserum, and a  $^3\text{H}$  labelled ecdysone ligand with specific activity of 6Ci/mmol, detection of 200pg of 20-HE was achieved. This antiserum was found to have only slightly different affinity for ecdysone and 20-HE. However, a second antiserum was produced against a different immunogen (see Table 3.2) and found to bind ecdysone and 20-HE with relative affinities of 10:1 (Horn et al , 1976). These two sera allowed Chang et al (1976) to determine separately the amounts of each of these two ecdysteroids in mixed samples.

Other ecdysteroid RIAs have been reported (De Reggi et al , 1975; Porcheron et al , 1976; Lauer et al , 1974; Reum and Koolman, 1979). Of these, the former two used an iodinated radioligand, and the latter two used tritium. Table 3.2 gives some details of these and some

TABLE 3.2: Some properties of radioimmunoassays used for the quantitation of ecdysteroids.

Immunogen	Labelled Hapten	Separation method	Detection limit	RA
20-HE coupled to BSA	$^3\text{H}$ 20-HE (6Ci/mmol)	Salt precipitation	200pg ecdysone	1:1 <sup>a</sup>
Ecdysone coupled to thyroglobulin	$^3\text{H}$ ecdysone (60Ci/mmol)	Salt precipitation	20pg ecdysone	10:1 <sup>b</sup>
20-HE coupled to HSA	$^{125}\text{I}$ 20-HE	Equilibrium dialysis	10pg 20-HE	1:1 <sup>c</sup>
20-HE coupled to HSA	$^3\text{H}$ 20-HE (6Ci/mmol)	Salt precipitation	80pg 20-HE	20:1 <sup>d</sup>
20-HE coupled to HSA	$^3\text{H}$ 20-HE (50Ci/mmol)	Sephadex filtration	70pg 20-HE	1:1 <sup>e</sup>
Polypodine B coupled to RSA	$^3\text{H}$ ecdysone (68Ci/mmol)	Salt precipitation	80pg ecdysone	1:1 <sup>f</sup>
20-HE coupled to BSA	$^{125}\text{I}$ or $^3\text{H}$ 20-HE (3.4Ci/mmol)	Charcoal absorption	$\approx$ 5pg or $\approx$ 20pg 20-HE	na <sup>g</sup>
Inokosterone-26-oic acid coupled to thyroglobulin	$^3\text{H}$ 20-HE (68Ci/mmol)	Salt precipitation	$\approx$ 100pg ecdysone	1:0.3 <sup>h</sup>

BSA; bovine serum albumin, HSA; human serum albumin, RSA; rabbit serum albumin. RA; Relative affinity, ecdysone:20-HE.

<sup>a</sup>; Borst and O'Connor, 1972. <sup>b</sup>; Horn et al, 1976, and Reum and Koolman, 1979. <sup>c</sup>; DeReggi et al, 1975. <sup>d</sup>; Lauer et al, 1974. <sup>e</sup>; Garen et al, 1977. <sup>f</sup>; Maroy et al, 1977. <sup>g</sup>; Porcheron et al, 1976. <sup>h</sup>; Spindler et al, 1978.

other ecdysteroid RIAs. Reported RIAs for ecdysteroids are reviewed by Hirn and DeLagge (1980).

### Radioimmunoassay theory

The essence of RIA is the consistent stoichiometry of the interaction of an immunoglobulin with its antigen. The assay procedure involves the incubation of the two species in pre-determined proportions such that 50% of the antigen becomes bound to the antibody. If the immunoglobulin is then removed from the mixture, 50% of the antigen is removed still bound to it. The antigen is labelled, usually radiochemically, so that the relative amounts of antigen bound to the antibody and unbound in the incubation medium can be measured after this separation step (steps 1 and 2 in Fig. 3.1).

If an amount of unlabelled antigen is added to the incubation mixture prior to separation, it will compete with the labelled antigen and displace a proportion of it from the antibody binding sites (step 4, Fig 3.1). For a given amount of unlabelled antigen, the proportion of

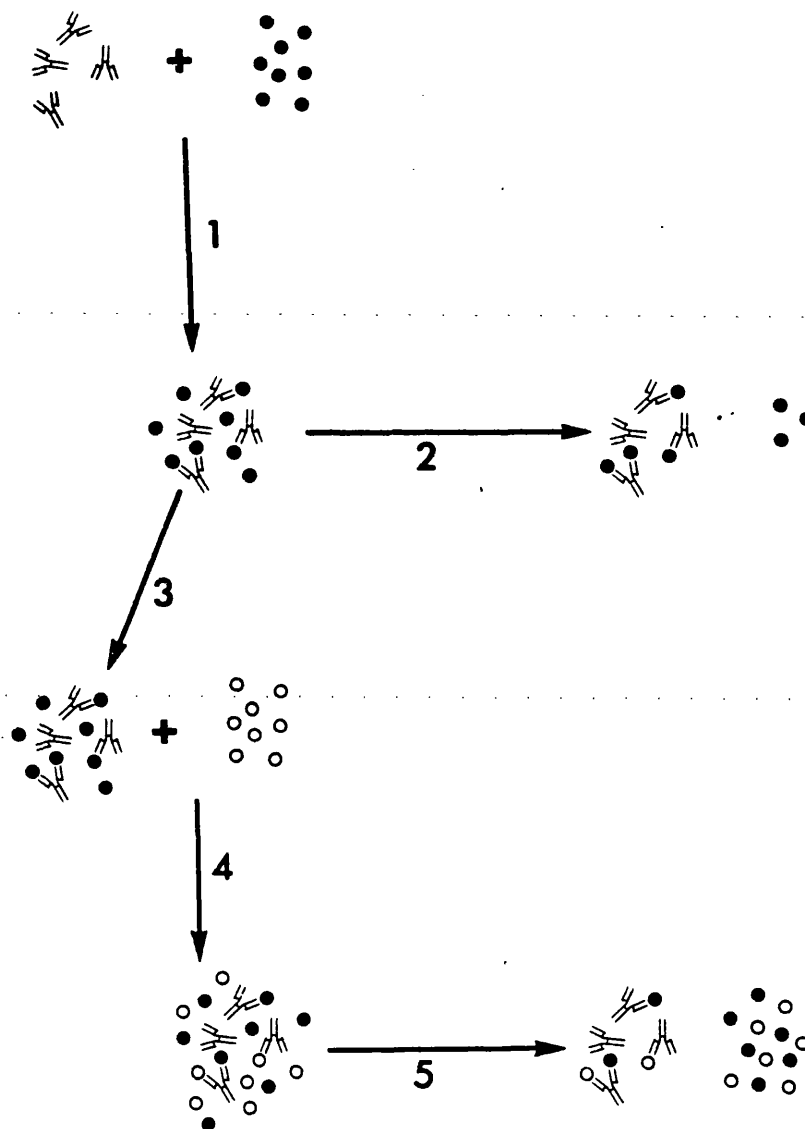


FIG. 3.1: DIAGRAMMATIC REPRESENTATION OF RIA THEORY. ANTIBODY ( $\text{Y}$ ) AND LABELLED ANTIGEN ( $\bullet$ ) ARE MIXED (STEP 1), SUBSEQUENT SEPARATION YIELDING 50% OF THE LABEL BOUND TO THE ANTIBODY (STEP 2). ADDITION OF UNLABELLED ANTIGEN ( $\circ$ ) TO THE MIXTURE PRIOR TO SEPARATION (STEP 3) DISPLACES SOME OF THE LABELLED COMPOUND (STEP 4) AND SUBSEQUENT SEPARATION REVEALS A DECREASE IN BOUND LABEL (STEP 5).

labelled antigen displaced from the antibody molecules will be constant, assuming the amounts of antibody and labelled antigen are constant. Subsequent separation of the bound and unbound fractions allows determination of the amount of labelled antigen which has been displaced. Theoretically, the relative affinities of antiserum binding to labelled and unlabelled antigen are unimportant, so long as the assay is standardised with known quantities of the unlabelled antigen. In practice, if the labelled and unlabelled antigens differ too much in their binding affinities, the usefulness of the assay is compromised in terms of sensitivity (if the labelled antigen binds with higher affinity) or range (if the unlabelled antigen binds with higher affinity).

#### Radioimmunoassay methodology

Three antibodies to ecdysteroids were available for use at the beginning of this project. Two of these (W1 and W2) were gifts from Dr. D L Whitehead (Tsetse Research Laboratory, University of Bristol), the other (H) from Dr. J D

O'Connor (Department of Zoology, University of California, Los Angeles). The avidities of the three antisera for ecdysone were compared, and the maximum sensitivity of ecdysone measurement determined (see below).

The choice between  $^{125}\text{I}$  and  $^3\text{H}$  as radiolabels for the RIA was made on the basis of maintenance of ligand binding and optimal radiolabel half life. Whilst use of  $^{125}\text{I}$  enables synthesis of high specific activity radioligand, which enhances the sensitivity of the assay, the effect of the relatively large iodine atom on the antigenicity of the ecdysteroid is not predictable. Also, the short half life of  $^{125}\text{I}$  (60 days) necessitates frequent resynthesis of labelled steroid.  $^3\text{H}$  ecdysone is available commercially at sufficiently high specific activity to produce a RIA of adequate sensitivity (see below). It has the benefits of being chemically identical to the unlabelled ligand with which it competes in the assay, and has a long half-life (12.3 years). On the basis of these arguments,  $^3\text{H}$  ecdysone (80Ci/mmol, New England Nuclear) was used in this study.



Steroid RIAs have the advantage over RIA of larger molecules of relatively easy separation of bound from unbound label, and a number of different methods have been used in ecdysteroid assays. These include absorption of free steroid by activated charcoal (Porcheron et al, 1976), separation by equilibrium dialysis (DeReggi et al, 1975), gel filtration (Garen et al, 1977) and ammonium sulphate precipitation (Borst and O'Connor, 1972). This latter technique has been found preferable on the basis of ease and reliability (Reum and Koolman, 1979), and so was chosen for use in this study.

#### Radioimmunoassay procedure

The assay procedure employed in this project was modified from the specifications of Borst and O'Connor (1972). All reagents used were of "Analar" grade (BDH), unless otherwise specified. Assay procedures were carried out in freshly made borate buffer (6.18g boric acid ( $H_3BO_3$ ); 9.5g borax ( $Na_2B_4O_7$ ); 4.39g sodium chloride (NaCl) in 1L distilled  $H_2O$ , pH 8.4).  $^3H$  ecdysone was stored in distilled  $H_2O$  at  $-20^\circ C$ . Prior to use

it was dissolved in borate buffer and the activity adjusted to  $4 \times 10^4$  cpm/ml. 100 $\mu$ l of  $^3\text{H}$  ecdysone solution was added to the dried sample (see below) in a 1.5ml Eppendorf conical centrifuge tube (type 3810). Samples were mixed thoroughly (about 5 seconds on a vortex mixer) to dissolve the dried sample, and 100 $\mu$ l of diluted antiserum was added. Antisera were diluted in 1% RIA grade BSA (Sigma) in borate buffer. The tubes were incubated at 4°C overnight. Precipitation of immunoglobulin was achieved by adding 200 $\mu$ l of saturated ammonium sulphate (SAS) in borate buffer at 4°C. Tubes were mixed thoroughly as soon as the SAS was added, and were allowed to stand for 20 to 30 minutes. The precipitate was pelleted by centrifugation on a Beckman J6 centrifuge, fitted with a JS-4.2 swinging bucket rotor, at 4500 rpm (about 5000g) for 1 hour at 4°C. Supernates were removed with a pulled Pasteur pipette attached to a water-driven suction pump, and 400 $\mu$ l of 50% SAS at 4°C added to wash the pellet. The tubes were mixed briefly, and the suspension pelleted again as described.

To count the amount of  $^3\text{H}$  ecdysone bound to the precipitated immunoglobulin, the supernate was

removed and the washed pellet suspended in 100 $\mu$ l of distilled H<sub>2</sub>O. 1ml of "Unisolve" liquid scintillator (Koch-Light) was added to each sample, and the Eppendorf tubes were placed within polythene scintillation tubes for counting. All counts were performed in a Phillips automatic liquid scintillation counter, using an external <sup>3</sup>H standard. No quench correction was applied, as a trial experiment showed no significant change in cpm recorded with volumes of water varying between 80 $\mu$ l and 120 $\mu$ l, in the counting regime described. The counting efficiency was 30%.

#### Preparation of standards

A small quantity (approximately 20 $\mu$ g) of ecdysone (Simes, Milan) was dissolved in "Puriss" grade ethanol (BDH) and the uv absorbance of the solution measured at 242nm using a Cecil 272 spectrophotometer. The concentration of ecdysone was calculated using the formula:

$$C = \frac{464 \times A_{242}}{12.4}$$

where C = concentration of ecdysone in  $\mu$ g/ml

$A_{242}$  = absorbance of solution at 242nm

12400 = Molar absorbance of ecdysone at 242nm

The concentration was adjusted to 100ng/ml, and the entire range of standards was measured from the single solution. Standards generally ranged from 0.5ng to 10ng, although on some occasions the range was extended below 0.5ng, when a second dilution of the 100ng/ml solution was made. The standards were dried at 40°C under N<sub>2</sub> in Eppendorf 1.5ml microfuge tubes, and stored in a dessicator at -20°C. For each assay performed, a range of standards was included in duplicate.

#### Standard variability

In order to determine the variation to be anticipated at a point on the standard curve, due to variability in standard preparation and in assay procedure (ie. intra-assay variability), 10 standards were assayed for each point on the standard curve, from 0.5 to 10ng. The results are given in Table 3.3 and in Fig. 3.2.

The observed cpm scores were regressed against the Log of the corresponding ecdysone

TABLE 3.3: RIA of ecdysone standards.

Ecdysone (ng.)	Recorded cpm <sup>1</sup>
10	696 +/- 17
5	866 +/- 23
3	1176 +/- 28
2	1514 +/- 49
1.5	1741 +/- 41
1	2013 +/- 7
0.5	2315 +/- 30

<sup>1</sup>; Counts given as mean +/- standard deviation of 10 determinations.

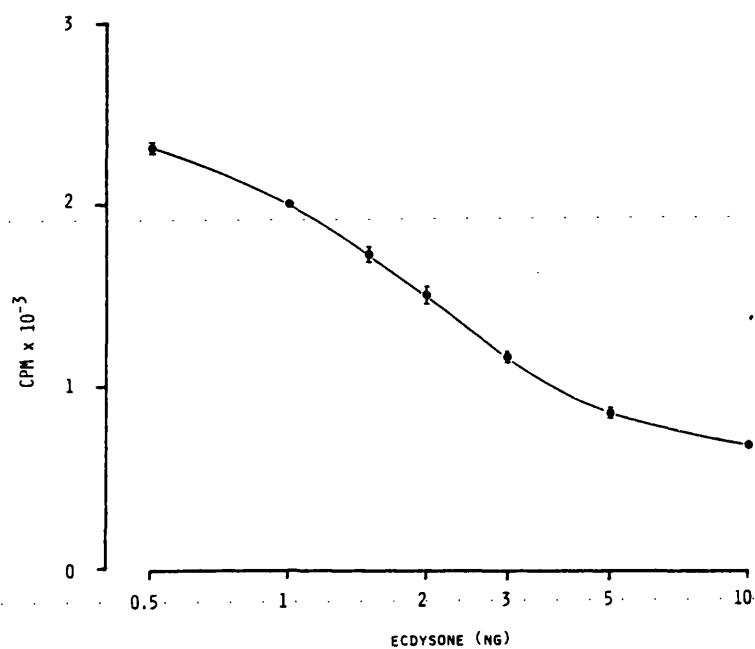


FIG. 3.2: STANDARD CURVE FOR ECDYSONE RADIOIMMUNO ASSAY. EACH POINT REPRESENTS MEAN AND STANDARD ERROR OF 10 ASSAYS.

standard values, and the Pearson correlation coefficient (r) found to be 99.5%. The standard deviation of the cpm scores about the regression line was 39.51, and from this the standard error of estimate can be calculated using the formula:

$$sy' = sy(1-r_{xy}^2)^{\frac{1}{2}}$$

where sy' - standard error of estimate

sy = standard error of y (the cpm scores)

$r_{xy}$  = Pearson correlation coefficient  
between x and y.

For further explanation, see Welkowitz et al ,  
1982.

In this case, sy' = 3.75, and this is small relative to sy (39.51), indicating that prediction errors will be small.

### Radiolabelled ecdysone

To check the purity of the labelled ecdysteroid preparation, it was co-chromatographed with ecdysone (Simes) using thin layer chromatography (TLC) on silica gel plates (Merck, West Germany). Prior to use, the plates were

heated to 100°C overnight. Samples were applied in 5 to 10µl of methanol, via a small bore polythene tube, so that the spot produced was of about 1.5mm diameter. Plates were developed using a 4:1 chloroform:methanol solvent system. The front was allowed to run 15cms from the origin, and the plate dried and scraped to 30 x 5mm fractions. Scrapings were eluted with 2mls of ethanol and the silica removed by centrifugation. 100µl of each fraction was dried under N<sub>2</sub> at 40°C, dissolved in 1ml distilled H<sub>2</sub>O, added to 10mls "Unisolve" and counted in a liquid scintillation counter. The uv absorbance at 242nm of the remainder of each fraction was also measured.

Fig. 3.3 shows the chromatogram of 250µg of ecdysone to which was added  $5 \times 10^5$  cpm <sup>3</sup>H ecdysone. The sample was loaded in 10µl of methanol. Although recovery of ecdysone was poor (about 31%), both <sup>3</sup>H ecdysone and ecdysone ran to the same position on the plate. As ecdysone and 20-HE can be resolved into separate peaks by TLC using this solvent system (Morgan and Poole, 1976), this result implies neither the <sup>3</sup>H



ecdysone nor ecdysone preparations had major ecdysteroid contaminants.

This procedure was used to check both batches of  $^3\text{H}$  ecdysone used in this work. After the purity had been checked,  $^3\text{H}$  ecdysone was dissolved in distilled  $\text{H}_2\text{O}$ , divided into aliquots and stored at  $-20^\circ\text{C}$ .

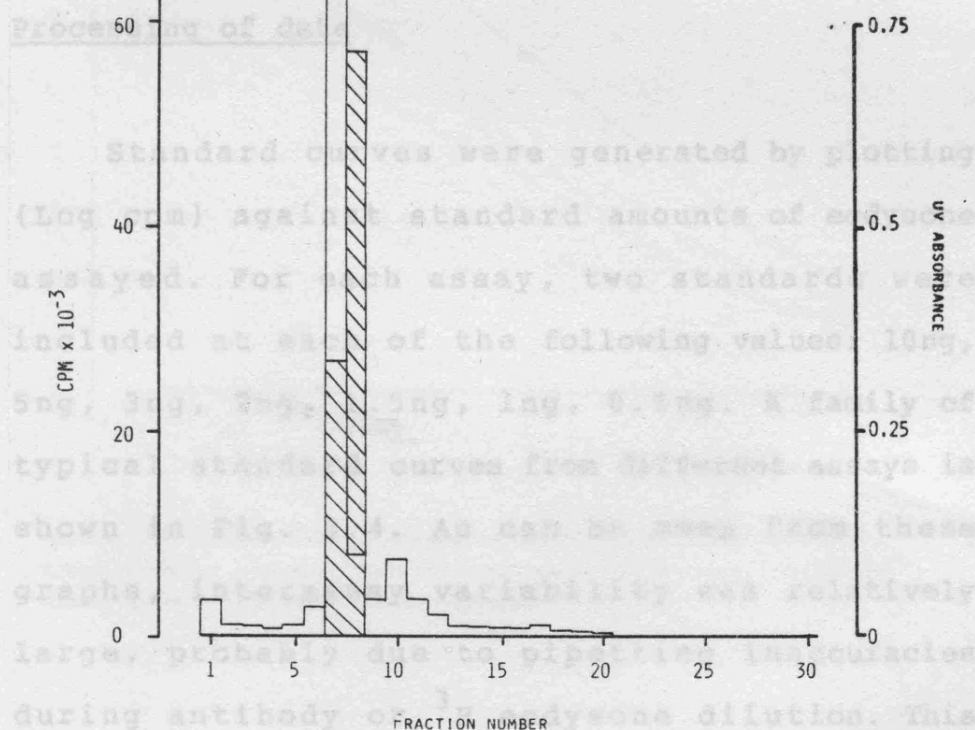


FIG. 3.3: RADIOCHROMATOGRAM OF  $^3\text{H}$  ECDYSONE. ECDYSONE (SHADED BARS) AND  $^3\text{H}$  ECDYSONE WERE CO-CHROMATOGRAPHED BY TLC ON SILICA GEL PLATES.

ecdysone nor ecdysone preparations had major ecdysteroid contaminants.

This procedure was used to check both batches of  $^3\text{H}$  ecdysone used in this work. After the purity had been checked,  $^3\text{H}$  ecdysone was dissolved in distilled  $\text{H}_2\text{O}$ , divided into small aliquots and stored at  $-20^\circ\text{C}$ .

#### Processing of data

Standard curves were generated by plotting {Log cpm} against standard amounts of ecdysone assayed. For each assay, two standards were included at each of the following values: 10ng, 5ng, 3ng, 2ng, 1.5ng, 1ng, 0.5ng. A family of typical standard curves from different assays is shown in Fig. 3.4. As can be seen from these graphs, interassay variability was relatively large, probably due to pipetting inaccuracies during antibody or  $^3\text{H}$  ecdysone dilution. This necessitated construction of a separate standard curve for each assay performed. The portion of the standard curve most closely resembling a straight line was selected by discarding results at the extremes of the range. A regression equation for

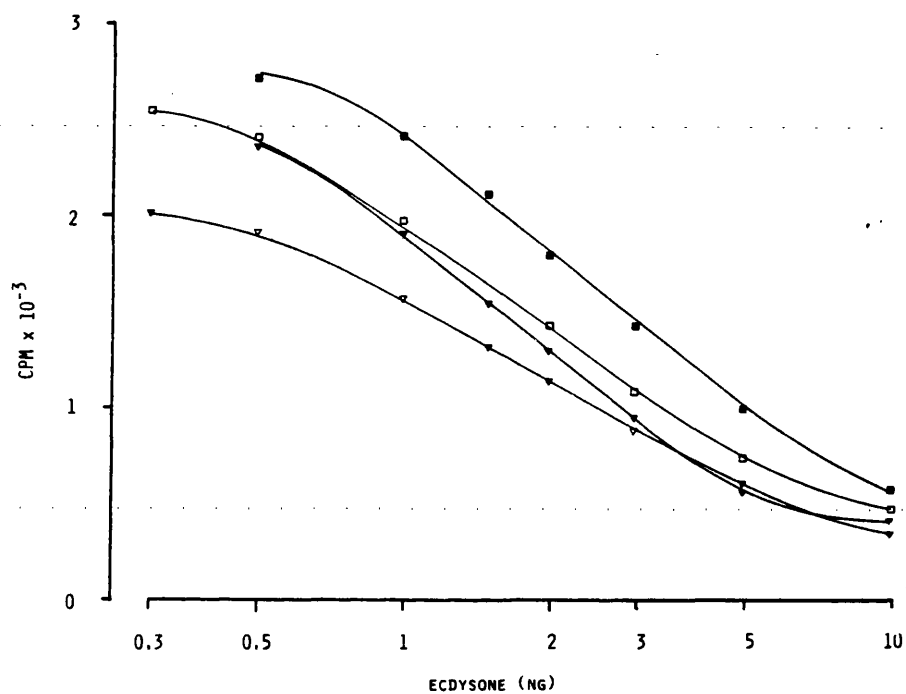


FIG. 3.4: STANDARD CURVES FOR ECDYSONE RADIOIMMUNOASSAYS. POINTS REPRESENT MEANS OF 2 DETERMINATIONS. THE SHAPE OF THE CURVE VARIED CONSIDERABLY BETWEEN ASSAYS, NECESSITATING CONSTRUCTION OF A NEW STANDARD CURVE FOR EVERY ASSAY.

the points selected was generated by the least squares method (Welkowitz et al , 1982). The regression equation was used to determine quantities of ecdysone in the experimental samples. Values for the Pearson correlation coefficient ( $r$ ), calculated as a measure of the goodness of fit of the transformed standard curve to the regression line, were 0.97 to 0.99 and never less than the lower value. Such high values for  $r$  (which may only vary between +1 and -1) imply very little error will be involved in computing values of ecdysone from measured cpm (see Welkowitz et al , 1982, pp 194-195).

#### Preparation of samples

Tissue culture medium was prepared for assay by adding an equal volume of methanol, mixing thoroughly, and centrifuging to remove the precipitate. The supernate was pipetted to 1.5ml Eppendorf microfuge tubes in a range of fractions, usually 20%, 10% and 5% of the total. All samples were assayed in duplicate. The methanolic extracts were dried at 40°C under  $N_2$  and stored at 4°C prior to assay. A small number of measurements

performed on extracts to which known amounts of unlabelled ecdysone had been added showed that the assay provided a quantitative measurement of ecdysone in these extracts.

In order to check further that a true measurement of ecdysone was being obtained, a number of samples were further purified, using the following procedure: methanolic extracts were washed with hexane to remove lipids, and partitioned three times against water-saturated butanol. In this system, ecdysone goes to the butanol phase, and this was evaporated under low pressure at room temperature in a rotory evaporator. This procedure did not reduce the ecdysone content of the samples as measured by RIA. From these experiments it was concluded that RIA performed on the single-step methanolic extracts provided a reliable measure of ecdysone in tissue culture medium.

A small number of whole animal extracts were also assayed for ecdysone. These were prepared by washing Manduca larvae in tap water, and homogenising the entire animal in about 25mls of 50% methanol. The homogenate was mixed on a vortex mixer and centrifuged for 10 minutes at 1000g to

remove the precipitate. The pellet was washed once in 50% methanol and recentrifuged. The supernate was added to the sample, and the total volume measured. Aliquots of various sizes (usually from 1 to 0.05% of the total volume) were dried under N<sub>2</sub> in Eppendorf microfuge tubes and assayed directly. The validity of this procedure was verified by adding to some of the samples known amounts of ecdysone as described for samples of tissue culture medium. The efficiency of the extraction was tested by injection of some animals with about  $2.5 \times 10^5$  cpm of <sup>3</sup>H ecdysone in 100μl of Manduca saline less than 10 minutes prior to the extraction step. Subsequent measurement of <sup>3</sup>H in the extract showed the procedure to be better than 95% efficient for ecdysone.

#### Comparison of three ecdysone antisera

The ecdysone binding characteristics of the three antisera (H, W1 and W2) were compared. Antiserum H is similar to that described by Horn et al (1976), although it may be a different bleed from the same animal. Antisera W1 and W2

represent separate bleeds from a rabbit immunised with 20-HE-carboxy-methyloxime conjugated to BSA (D L Whitehead, personal communication).

Serial dilutions of the three antisera were prepared and assayed using the protocol described above, in order to determine the antiserum dilution at which binding of 50% of the  $^3\text{H}$  ecdysone occurred (Fig. 3.5). Whilst W1 bound 50% of the labelled steroid at higher dilution than either of the other two antisera, it was available only in limited amounts. To ensure sufficient antiserum for all the assays reported here, W2 was used routinely for this project. Further assays showed that a dilution of 1:300 consistently gave 50% binding when  $4 \times 10^3$  cpm of  $^3\text{H}$  ecdysone were added. It should be noted that due to the assay procedure, the final antibody dilution in the assay is 1:600.

Standard curves were constructed for all three antisera at the dilutions giving 50%  $^3\text{H}$  ecdysone binding (Fig. 3.6). It can be seen that the curve for W2 gives a linear relationship for standard amounts of ecdysone between 1 and 5ng. The experiment was repeated with values for ecdysone standards from 0.2 to 20ng (Fig. 3.7).

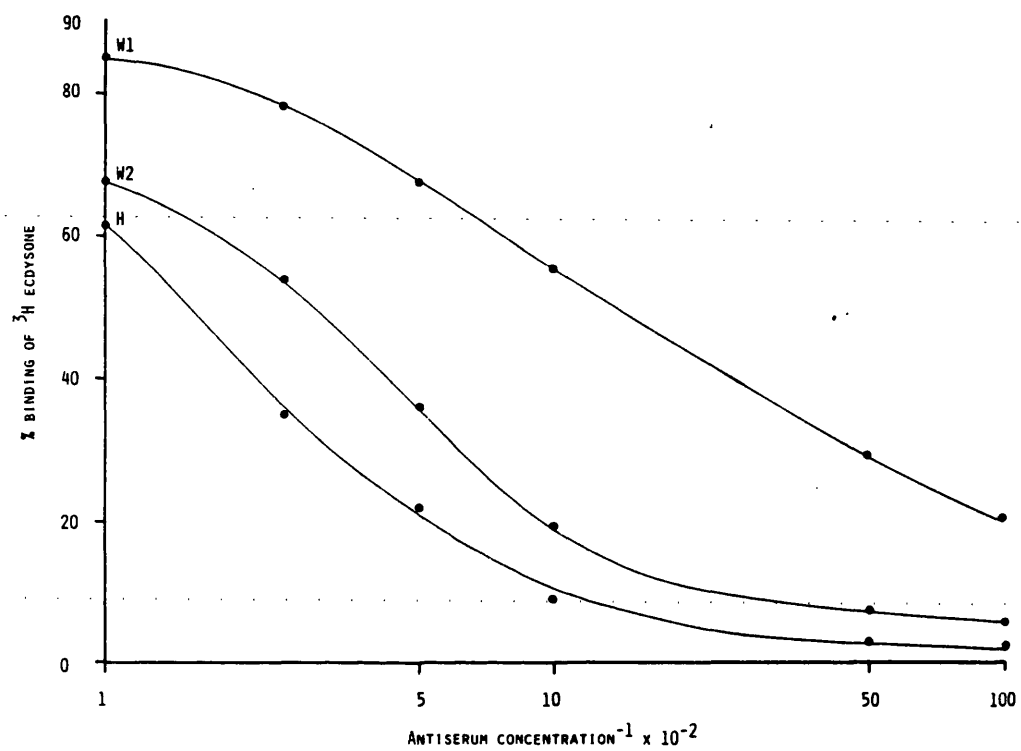


FIG. 3.5: COMPARISON OF BINDING OF <sup>3</sup>H ECDYSONE BY VARYING CONCENTRATIONS OF 3 DIFFERENT ANTISERA. POINTS REPRESENT MEANS OF TWO DETERMINATIONS. SEE TEXT FOR DETAILS.



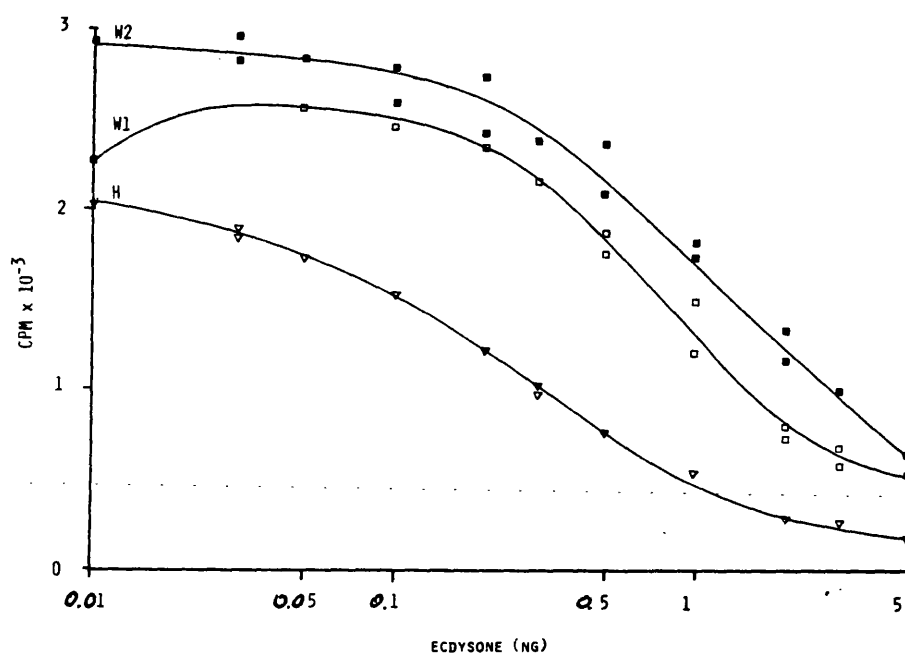


FIG. 3.6: COMPARISON OF ECDYSONE RADIOIMMUNOASSAY STANDARD CURVES FOR 3 DIFFERENT ANTISERA. ALL DETERMINATIONS WERE MADE IN DUPLICATE. WHERE A SINGLE POINT APPEARS, BOTH DETERMINATIONS FALL WITHIN THIS POINT. EACH ANTISERUM WAS USED AT THE CONCENTRATION GIVING 50% BINDING OF THE UNLABELLED STEROID.

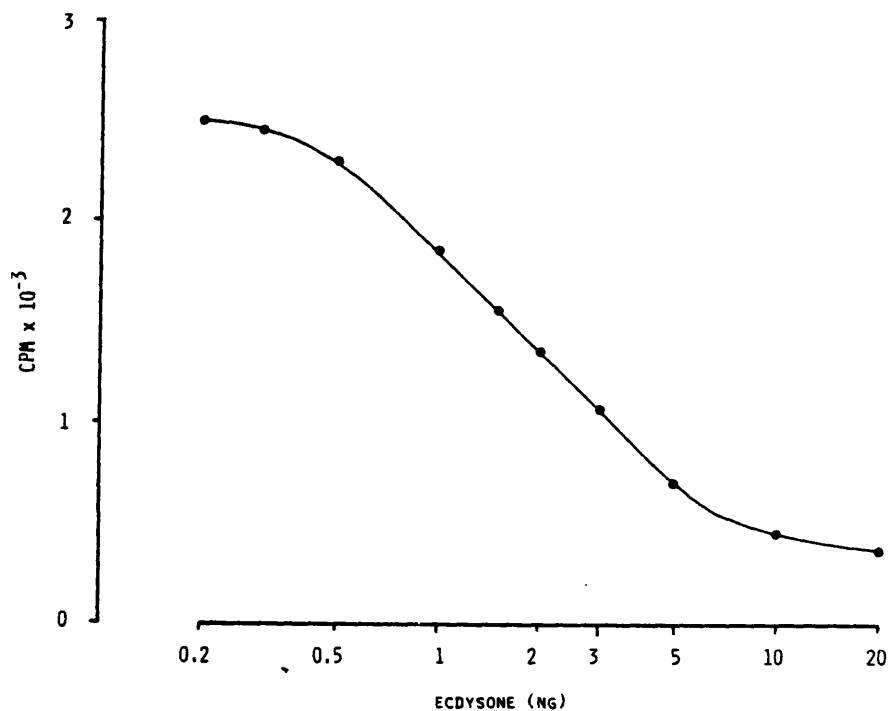


FIG. 3.7: EXTENDED ECDYSONE RADIOIMMUNOASSAY STANDARD CURVE FOR W2 ANTISERUM. POINTS REPRESENT MEANS OF 2 DETERMINATIONS.

The result shows a usable portion of the curve (ie. approximating to a straight line) between 1 and 5ng, and this was usually observed in subsequent assays.

#### Relative affinity of W2 for ecdysone and 20-HE

It was assumed that the major ecdysteroid present in whole animal extracts in addition to ecdysone would be 20-HE (see Introduction). In order to investigate the activity of 20-HE in the assay, an experiment was conducted to measure a range of both ecdysone and 20-HE (Simes) in the same assay (Fig. 3.8). As can be seen from the graphs, and Table 3.4, 20-HE displays activity ranging from 106% (at 1ng) to 71% (at 5ng) that of ecdysone. The observation that standard curves for ligands of different relative affinities have different slopes is common (Ekins, 1974). These results imply that measurement of ecdysteroid content of samples containing 20-HE will be in error by, at most, 29%. This amount of error was considered reasonable in view of the small number of measurements to be made on whole animals and these not being central to this project. In the

worst possible case, where 100% of whole animal ecdysteroid is 20-HE, ecdysteroid content of whole animals will be under estimated by about 30%.

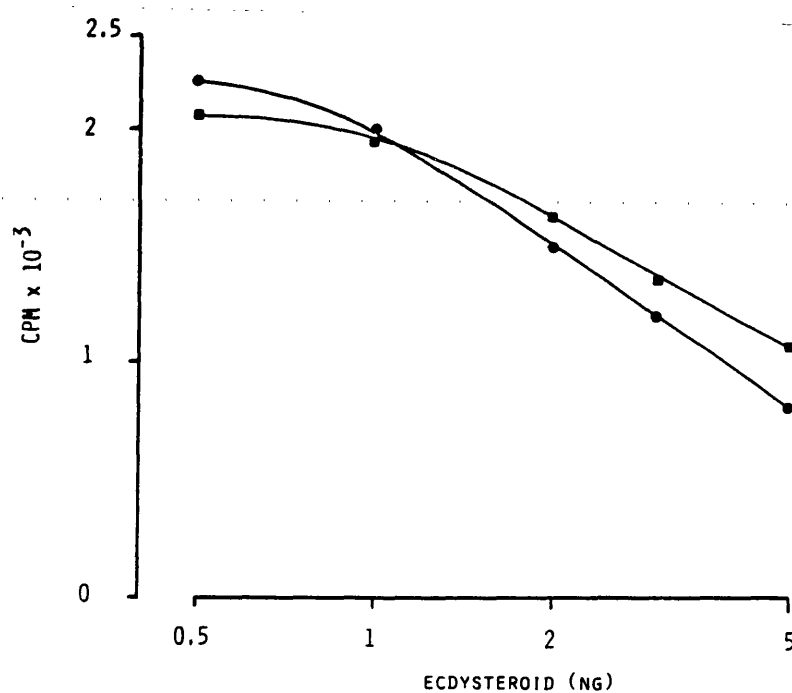


FIG. 3.8: ACTIVITY OF ECDYSONE (●) AND 20-HE (■) IN ECDYSONE RADIOIMMUNOASSAY, USING W2 ANTISERUM. POINTS REPRESENT MEANS OF THREE DETERMINATIONS.

TABLE 3.4: Activity of 20-HE relative to that of ecdysone  
in RIA.

20-HE standards (ng)	Activity in assay (ng ecdysone equivalent)	% ecdysone activity of 20-HE
5	3.58	71
3	2.44	81
2	1.79	89
1	1.06	106

## CHAPTER 4

### THE PROTHORACIC GLAND INCUBATION SYSTEM.

The in vitro maintenance of insect tissue is performed in a manner similar to that for other tissues, with necessary adjustments to conditions and media to satisfy differences in physiological requirements. As is the case with all tissue culture, the object is to imitate sufficiently closely the whole animal environment, so that the physiology of the tissue in vitro is comparable to that in vivo. Thus, the medium used is designed to mimic the extracellular fluid bathing the cells. Synthetic media have advantages over natural body fluids, in that they are defined and therefore not subject to variations beyond control of the investigator. The availability of synthetic media is also an advantage.

Tissue culture media are based on an inorganic salt solution designed to produce the correct osmotic pressure (op) and pH for maintenance of the excised tissue. Insect haemolymph exhibits considerable inter-specific variability in op, but in phytophagous insects,

such as are lepidopterous larvae, tends to be about double that of vertebrates, due to the contribution of organic molecules, especially amino acids. This latter property adds difficulty to measurement of op of Manduca haemolymph, which tends to be very viscous and rapidly coagulates.

Insect haemolymph has a pH of around 6.3 to 6.9, considerably lower than vertebrate blood, which is invariably of pH 7.4 to 8 due to its function in CO<sub>2</sub> transport (Wyatt, 1961). This low pH is reflected in the design of a culture medium. A special requirement of lepidopteran tissues is a high K<sup>+</sup> concentration, as this is the predominant cation of lepidopteran haemolymph. The basic salt solution is modified with supplements to meet these requirements.

Gaseous exchange of insect cells in culture is a considerable problem as, unlike vertebrate cells which undergo exchange with their surrounding fluid, they are provided in vivo with a tracheolar supply, difficult to imitate in vitro. The maintenance of the tracheolar system in excised tissue has seldom been achieved, (but see Truman, 1978) and most workers have

relied on the cells performing exchange with the bathing medium. It is possible, by modifying the atmosphere over the culture medium, to alter the concentrations of dissolved gasses. This has been shown to have beneficial effects in some long term insect tissue culture systems (eg, Chino, 1976).

First attempts at insect tissue culture were made by Goldschmidt (1916). An improvement in techniques allowed Trager (1935) to maintain Bombyx ovarian tissue in hanging drop cultures for up to two weeks. Once tissue culture became more widely recognised as a logical method to reduce the number of variables which act on a tissue in vivo, an explosion in the use of this technique for all tissues, including those of insects, occurred. (For reviews, see Jones, 1962; Schneider, 1967; and Marks, 1970.) A particularly successful early attempt at insect tissue culture was the maintenance of epidermal tissue from pupal wings and antennae of Samia walkeri (Drury) and Antherea polyphemus (Cram), for up to six weeks in vitro (Loeb and Schneiderman, 1956).

Advances in synthetic media simplified the in vitro technique. The medium used in this project is slightly altered from that used by

Grace (1958) for maintenance of silkworm tissues, being a modification itself, by the addition of some of the B vitamins, of Wyatt's (1956) medium. The full specification of this medium is given in Appendix A.

Grace's medium is a defined mixture and as such contains none of the macromolecular element found in haemolymph. Paul (1975) states that generally about 10 to 20% serum, in the form of hemolymph or fetal calf serum is added to insect tissue cultures, to provide the undefined macromolecular element of the cells' in vitro environment. In the case of insect haemolymph, the polyphenol-polyphenol oxidase system which causes darkening and clotting on exposure to air (Susman, 1949) must first be inactivated. This is often achieved by the addition of phenolthiourea (PTU), which inhibits the enzyme tyrosinase, but Wyatt (1956) has found PTU to be inhibitory to cell growth. Therefore, heat treatment, followed by centrifugation can be used to remove large proteins from the blood (Wyatt, 1961). Chino et al (1974) removed tyrosine and other small molecules from haemolymph by Sephadex G-25 filtration, thus preventing darkening. In this study, it was not



found necessary to add haemolymph extracts to the basic Grace's medium (see below).

#### Early attempts at prothoracic gland culture

A variety of methods has been used for the in vitro maintenance of PG. The success of such experiments is best determined by assessing the ability of the excised tissue to retain its normal function. First reports came from Oberlander et al (1965), who performed some preliminary experiments with PG from Philosamia ricini (Donovan) maintained in hanging drops of TC 199 medium. Activity of the PG was measured in terms of RNA synthesis, and an increase in synthetic activity in the presence of isolated brains was demonstrated. However, the results were confused by high level background RNA synthesis by PG incubated on their own. Other early attempts measured gland activity in vitro using bioassays for ecdysone based on a variety of ecdysone target tissues (Marks et al, 1968; Agui et al, 1972; Agui and Fukaya, 1973; Chino et al, 1974).

Chino (1976) considered cholesterol and oxygen to be important factors in PG maintenance. Using the Sarcophaga bioassay (Ohtaki et al, 1968) to measure ecdysone, he demonstrated that glands do not store synthesized ecdysone. He also showed that added haemolymph enhanced gland secretory activity, and demonstrated this to be due to the presence of cholesterol binding proteins (Chino and Gilbert, 1971), which were thought to enhance the ability of the gland cells to utilise cholesterol. However, the advent of a technique allowing direct measurement of secretory activity of individual PG in vitro, in the form of a RIA for ecdysone (Borst and O'Connor, 1972), enabled a much more accurate assessment of suitability of incubation conditions. The following discussion is limited to most recent reports of PG incubations.

#### Techniques for in vitro PG maintenance

Various aspects of incubation procedure may affect the performance of PG in vitro:

#### Choice of Culture Medium.

Chino et al (1974) found Wyatt's medium to be more suitable than Grace's for incubation of Bombyx PG. Ecdysone secretion over a period of three to five days was measured by the Sarcophaga assay. Supplementing the medium with a large molecule fraction of haemolymph, or with preparations of two cholesterol carrying lipoproteins isolated from haemolymph, increased secretory activity by up to 8 times (Table 4.1).

As these cultures were maintained over a considerably longer period than those attempted in this work, it is likely that endogenous supplies of substrate became exhausted, and the haemolymph supplement allowed enhanced utilisation of substrate from the medium. Substrate limitation seems less likely to be a limiting factor in short term incubations, as insect tissues characteristically have a high cholesterol content when removed from the animal (Gilbert and King, 1973).

Bollenbacher et al (1975) utilised a medium designed by Ittycheriah (1972), and supplemented it with macromolecules, as described by King et al (1974), to incubate Manduca PG. However,

TABLE 4.1: Secretion of ecdysone by Bombyx prothoracic glands incubated in various media (from Chino et al, 1974)

Medium	Gland number	Secreted ecdysone <sup>a</sup>
Graces	14	4.8
Wyatts	24	17.7
Haemolymph	25	120
Lipoprotein 1	21	148
Lipoprotein 2	16	108

<sup>a</sup> Ecdysone measured in ng 20-HE equivalent/gland pair.

Culture medium volume 0.04 - 0.06ml. Protein content of media : haemolymph 1.3mg/culture, lipoprotein 1, 0.54mg/culture, lipoprotein 2, 0.50mg/culture.

rates of ecdysone secretion, as measured by RIA were too low to account for the measured whole animal titres. Maximum recorded rates of about 65ng ecdysone/pair of PG/24 hours contrast with whole animal titres rising to 3.5µg ecdysteroid over two days.

In later work by the same group (Bollenbacher et al, 1979; Agui et al, 1979), Grace's medium has been used with similar results.

#### Gassing

Chino (1976) suggested PG in vitro require an excess of oxygen, and demonstrated an improvement in synthetic ability of Bombyx PG when cultures were maintained under a high O<sub>2</sub> atmosphere (Chino et al, 1974. Table 4.2). Perfusing the tracheal system with air is extremely difficult in the case of PG, as both the glands and their tracheal supply are very diffuse.

Other workers have maintained PG in an air atmosphere (King et al, 1974), and in the work described here no modification of the atmosphere over the medium has been found to be necessary.

TABLE 4.2: Effect of partial pressure  $O_2$  on ecdysone production by Bombyx prothoracic glands in vitro (from Chino et al, 1974).

Partial pressure $O_2$ (atm.)	Gland number	Secreted ecdysone <sup>a</sup>
0.2	15	25
0.5	25	97

<sup>a</sup> Ecdysone measured in ng 20-HE equivalent/gland pair, over 3-5 days.

### Volume of culture medium

Most workers have used a relatively small volume of culture medium in which to maintain PG. For example, Chino et al (1974) used standing drops of only 40 to 60 $\mu$ l. Agui et al (1979) and Bollenbacher et al (1979) use a mere 25 $\mu$ l of medium in hanging drop cultures. Volumes as small as these lead to evaporation problems, and prevent the gland cells spreading out their in vivo configuration. Previous work by the same group (King et al, 1974 and Bollenbacher et al, 1975) used 1.5 mls of medium per gland pair. This seems a more reasonable volume to use and, if the incubations are maintained in capped chambers, eliminates evaporation problems and hence the need for a high humidity atmosphere.

### PG incubation technique used in this project

Animals were removed from culture and anaesthetised by immersion in water for 30 minutes. Surface sterilisation was performed by immersion in 70% ethanol for 30 seconds, followed by two washes in sterile distilled water.

Dissections were performed in a sterile environment under Manduca saline (see Appendix B) and both PG were removed rapidly, avoiding rupture to the gut. Dissections could be performed in about 20 minutes per gland pair. Glands were immersed in fresh saline to wash off haemolymph and any unwanted tissue such as tracheolar branches was trimmed off. Glands were incubated individually in 1 or 1.5 mls of Graces tissue culture medium (pH 6.5, without added haemolymph or glutamine, Gibco), in 10 ml flat bottomed specimen tubes with oxoid caps. 1 ml of medium formed a layer just deep enough to cover the bottom of the tube. In experiments involving the addition of JH to the medium, tubes were siliconised (Repelcote, BDH) and 1 ml would no longer cover the whole base. 1.5 mls of medium was used in these experiments. Incubations were maintained at 28°C in an air atmosphere using a heated water bath.

Culture medium was removed from the culture vessels at periods from 1 to 6 hours and was replaced with fresh medium at 28°C. In the case of treatment with hormones, control and



experimental glands were rinsed twice in fresh medium before being re-incubated in fresh tubes.

Preparation of samples of medium for RIA is described elsewhere.

In vitro secretory rates of PG isolated at various times during the last larval instar

Using the incubation techniques described above, the rates of secretion of PG isolated from final larval stage Manduca were assessed. Animals were sacrificed at 24 hour intervals between the fifth larval ecdysis and pupal ecdysis. In accordance with the findings of Agui et al (1979), left and right glands from the same individual secreted at similar rates. For a sample of 48 paired secretions, left PG were found to secrete  $101 \pm 6.93\%$  (mean and standard error) of the amount of ecdysone secreted by right PG. Importantly, neither gland of a pair tends to secrete faster than the other, allowing paired glands from the same animal to be used for control and experimental observations.

Figures 4.1 to 4.7 show secretory rates of PG isolated from Manduca larvae at various times during the fifth instar. Glands from L5;0.17 animals show low rates of ecdysone secretion of about 1.5ng/hour, whilst those isolated from L5;1.17 and L5.2.17 individuals show similar higher rates of about 5ng/hour. These animals have not yet made the "decision to pupate" (Nijhout and Williams, 1974a), and these results are in agreement with the findings of other workers that prothoracic glands are continually active at a low level, and are never completely switched off (Bollenbacher, Vedeckis, Gilbert and O'Connor, 1975).

Glands from gate 1 L5;3.17 and gate 2 L5;4.17 animals secrete at much higher rates than those isolated earlier in the instar (around 13ng and 20ng/hour respectively). There is a significant difference ( $p \leq 0.01$ ) between these two rates of secretion (Table 4.3) which is discussed below. Secretion rates of glands from gate 2 L5;3.17 animals were not measured.

Whereas prewandering animals were sorted into gate 1 and gate 2 groups (see Chapter 2), animals which had already entered the wandering stage are

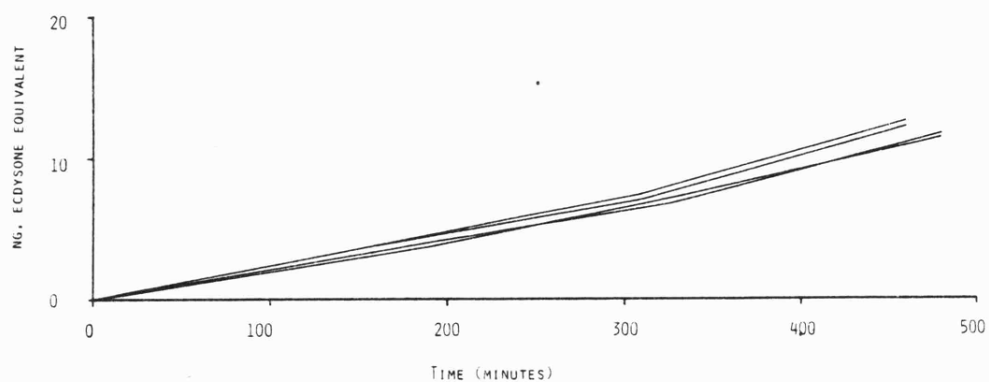


FIG 4.1: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED L5:0.17

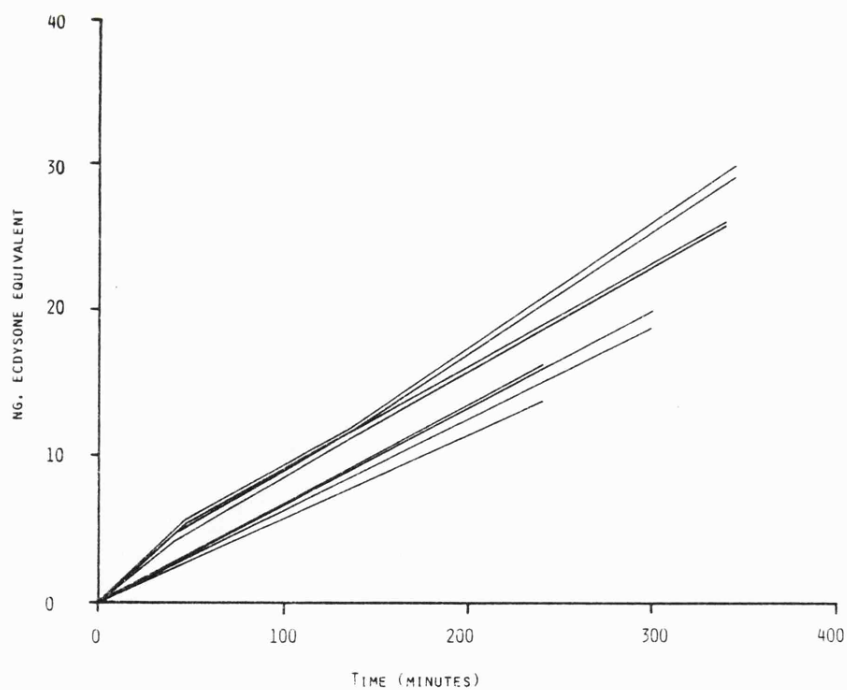


FIG. 4.2: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED L5:1.17

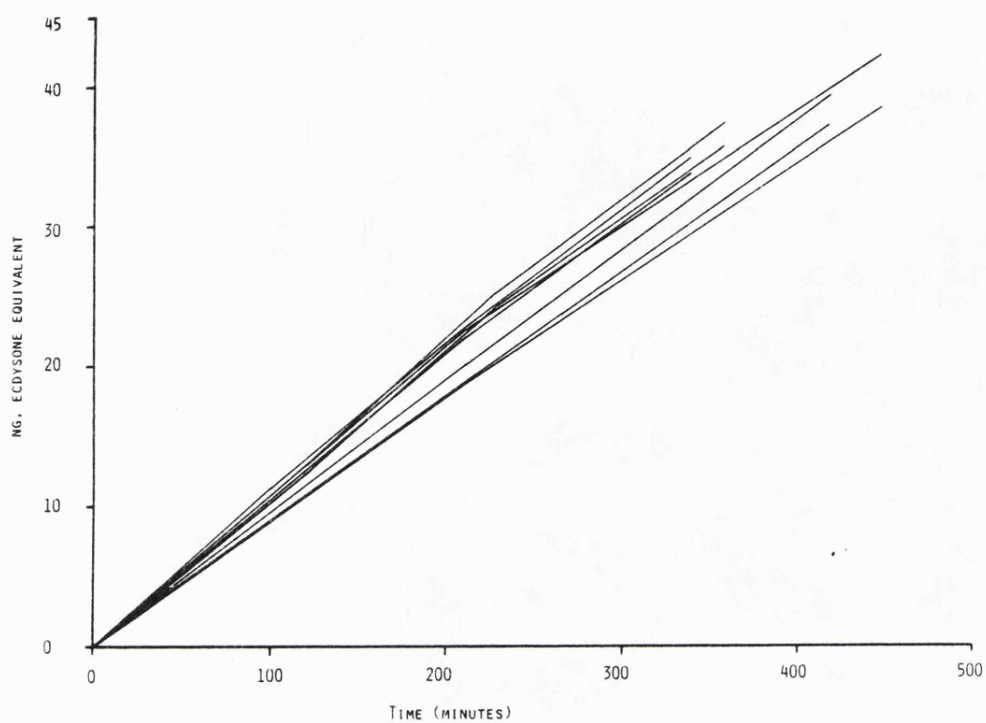


FIG. 4.3: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED L5:2.17

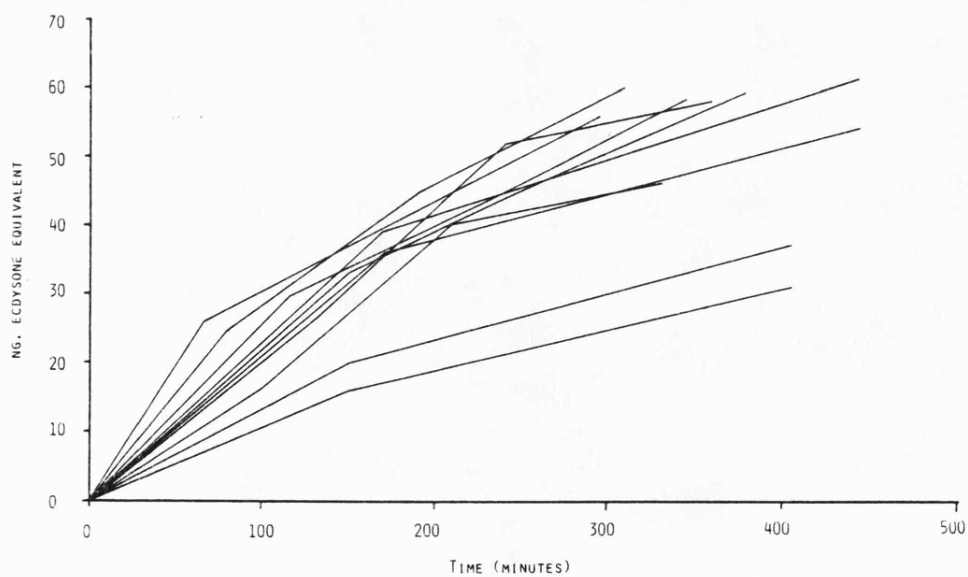


FIG. 4.4: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED L5:3.17 (GATE 1)

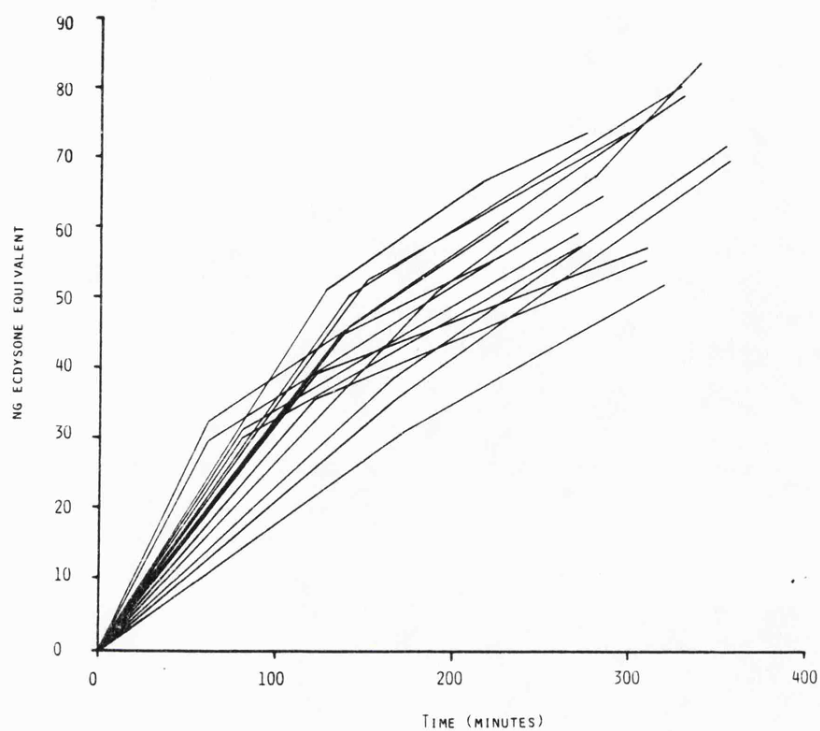


FIG. 4.5: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED L5:4.17 (GATE 2)

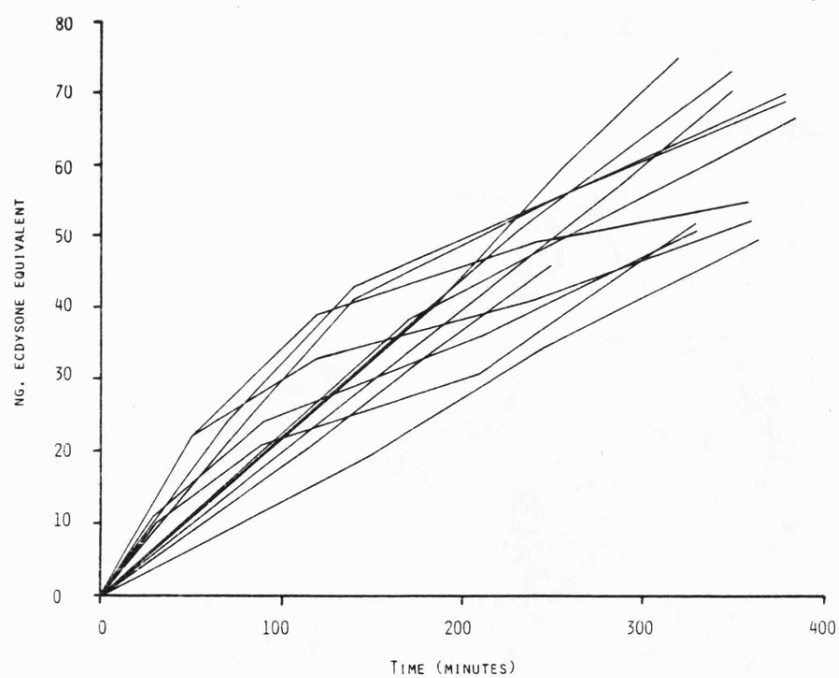


FIG. 4.6: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED EG:0.17

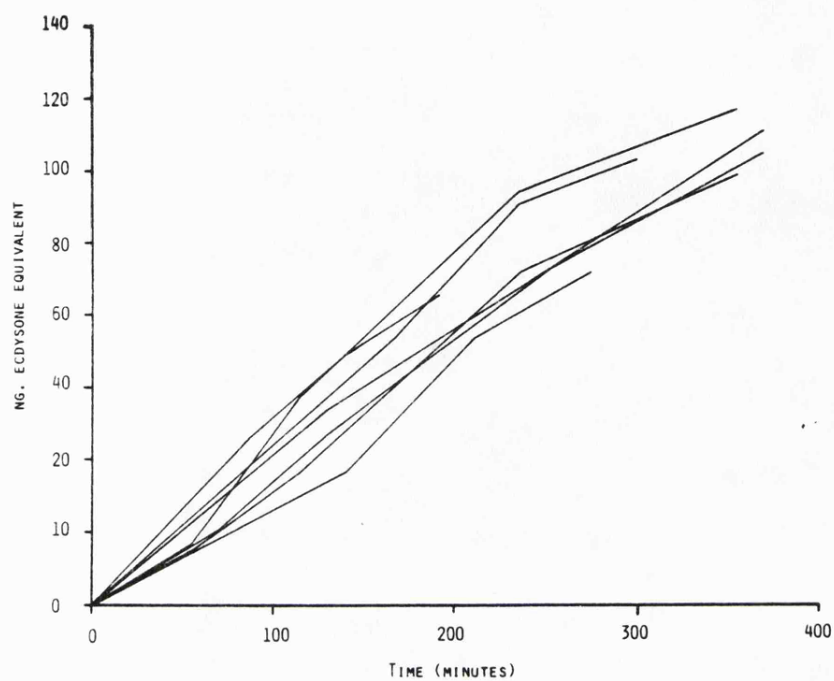


FIG. 4.7: IN VITRO SECRETORY RATES OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS AGED EG:1.17

TABLE 4.3: Initial secretory rates of prothoracic glands  
in vitro.

Age of donors	N	Ecdysone secretion (ng/hour) (mean +/- standard deviation)
L5;0.17	4	1.44 +/- 0.05
L5;1.17	8	4.94 +/- 0.41
L5;2.17	8	5.94 +/- 0.17
L5;3.17 (gate 1)	10	13.1 +/- 1.54 <sup>1,2</sup>
L5;4.17 (gate 2)	15	20.1 +/- 1.67 <sup>1,3</sup>
Eg;0.17	12	15.7 +/- 1.44 <sup>2,3,4a</sup>
Eg;1.17	7	21.5 +/- 2.14 <sup>4b</sup>

<sup>1</sup> - Significantly different ( $p \leq 0.01$ )

<sup>2</sup> - Not significantly different ( $p \geq 0.05$ )

<sup>3</sup> - Significantly different ( $p \leq 0.05$ )

<sup>4</sup> - Significantly different ( $4a < 4b$ ,  $p \leq 0.025$ )

All by t test (Welkowitz et al, 1982 p159)

a mixture of these two types. Thus, the results for Eg;0.17 animals are not significantly different from those for L5;3.17 ( $p \geq 0.05$ ) but are significantly different from those for L5;4.17 ( $p \leq 0.05$ ) animals. However, the range of secretory rates observed ( $15.7 \pm 1.44\text{ng}$ ,  $n=12$ ) is quite similar to that obtained if the secretory rates for L5;3.17 and L5;4.17 are combined ( $17.4 \pm 1.28\text{ng}$ ,  $n=25$ ). These rates are not significantly different, even at  $p=0.2$ . We can conclude from this that there is little change in PG activity between animals about to undergo wandering and those immediately after wandering. However, the secretory rate for glands from animals one day older is significantly different from animals immediately post-wandering ( $p \leq 0.025$ ) at  $21.5 \pm 2.14\text{ng}$ ,  $n=7$  for Eg.1.17 animals (Table 4.3). This implies that a second acceleration of ecdysone secretion by the PG has occurred between these two ages, which agrees with the timing of the two PTH releases postulated by Truman and Riddiford (1974).

Preliminary experiments with glands from animals aged Eg;2.17, that is post pupal apolysis, indicate that the rate of secretion has begun to



decline (five PG incubated for between 3 and 3.5 hours. had a mean secretory rate of  $15.8 \pm 2.21$  ng/hour).

#### Estimation of total in vivo ecdysone production

From the graphs of the secretions of PG from donors aged L5;0.17 to Eg;1.17, the hourly secretory rates over the initial period of incubation (between 1 and 3 hours) were estimated (Fig. 4.8). The graph shows a rise in secretory rate throughout the feeding stage, with a major increase between L5;2.17 and L5;3.17 for gate 1, and between L5;2.17 and L5;4.17 for gate 2 animals. This probably represents the response to PTH release from the brain at this time (Truman and Riddiford, 1974), and is reflected in the first (small) ecdysone peak seen in whole animal titres (Bollenbacher et al 1975, Fig. 4.9).

Estimations of the total amount of ecdysone which would be secreted in the time available, assuming constant secretion in vivo at these rates (ie. the area under the graphs in Fig. 4.8), agree reasonably well with my estimation of the

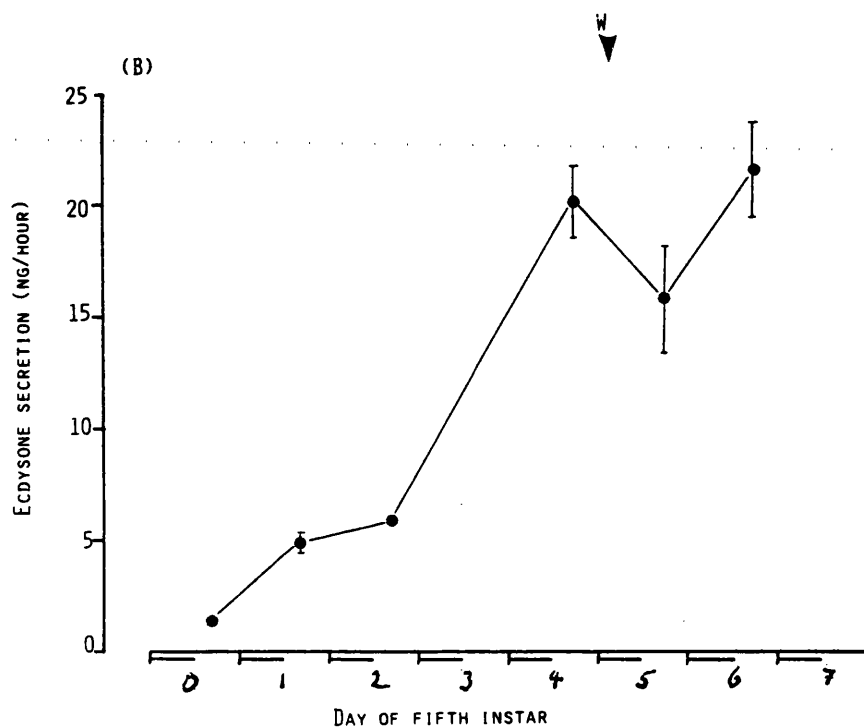
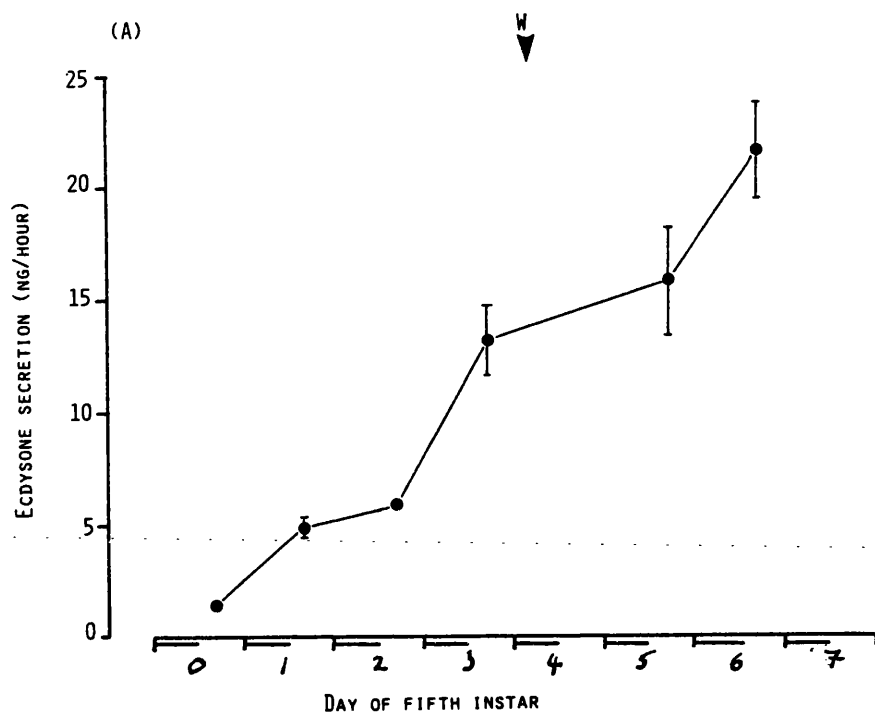


FIG. 4.8: INITIAL IN VITRO SECRETORY RATE OF PROTHORACIC GLANDS ISOLATED FROM ANIMALS OF VARIOUS AGES. (A) INCLUDES GATE 1 PRE-WANDERING LARVAE, (B) INCLUDES GATE 2 PRE-WANDERING LARVAE. W INDICATES TIME OF ENTRY TO WANDERING STAGE, POINTS REPRESENT MEANS WITH STANDARD ERRORS. SEE TABLE 4.3 FOR NUMBERS OF EXPERIMENTS PERFORMED AT EACH STAGE. BARS ON ABCISSA REPRESENT HOURS OF DARKNESS.

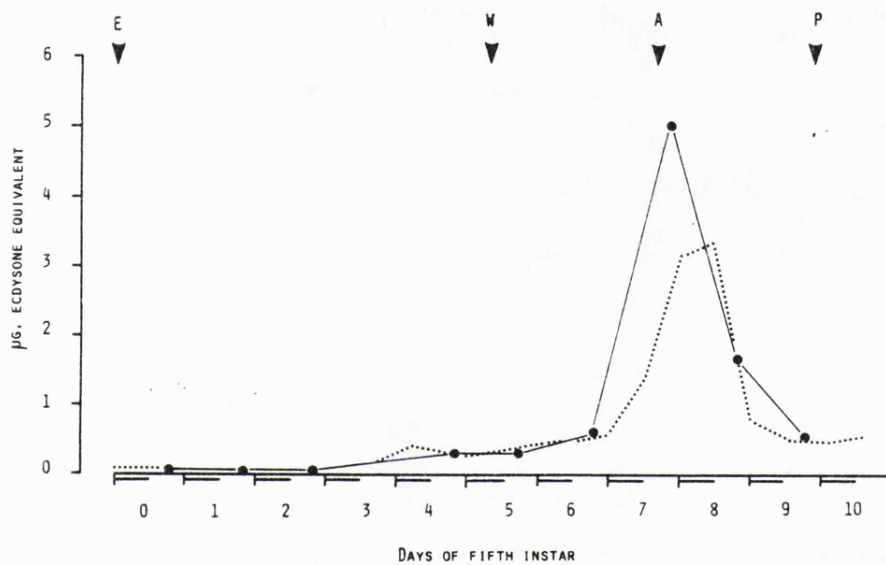


FIG. 4.9: WHOLE ANIMAL TITRES OF ECDYSTEROID MEASURED IN INDIVIDUAL ANIMALS DURING THE FIFTH INSTAR. ● REPRESENTS MEANS OF THREE INDIVIDUALS, EACH ASSAYED IN TRIPPLICATE. .... REPRESENTS VALUES GIVEN BY BOLLENBACHER ET AL (1975). E: ECDYSIS TO FIFTH INSTAR, W: WANDERING, A: APOLYSIS, P: PUPAL ECDYSIS. BARS ON ABSCISSA REPRESENT HOURS OF DARKNESS. FOR METHODS SEE CHAPTER 3.

total amount of ecdysteroid present in whole animals (Fig. 4.9). This in turn agrees very closely with that of Bollenbacher et al (1975), who determined whole animal ecdysteroid titres in insects raised under long day (ie. non-diapause inducing) conditions. In fact, there appears to be an excess of ecdysone secreted early in the instar, which may be rendered unavailable to the assay (and, perhaps, also to the target cell receptors) by binding to carrier molecules in the haemolymph, or by metabolic inactivation to methanol-precipitable or non-immunoreactive breakdown products.

#### Attempts to improve the culture system

The system described appears to allow the PG to continue secreting ecdysone at their anticipated in vivo rate for up to 6 hours after extirpation. Towards the end of this period, rates of secretion decline somewhat for the more active glands (Figs. 4.4 to 4.7), though less active ones continue to exhibit the same low rates for considerably longer.

In an attempt to prolong the period of high activity of rapidly secreting PG in vitro, a haemolymph fraction was added to the incubation medium. The possibility that either humoral promoters of gland activity or substrates for ecdysone synthesis were lacking, and so limiting gland activity, was thus investigated.

Haemolymph was drained from L5;2.17 Manduca larvae by removing one or two prolegs after anaesthesia. About 0.5ml of blood was obtained from each animal without applying any pressure. The blood was heated to 100°C for 5 minutes, allowed to cool and centrifuged for 10 minutes at 950g in an Eppendorf microcentrifuge. The supernatant, which had lost the ability to darken on exposure to air (see above), was added to the incubation medium directly.

In the experiment described in Table 4.4, 250µl of haemolymph extract and 750µl of Grace's medium were mixed for each of the experimental incubations. Contralateral control glands were maintained in Grace's medium alone. Active PG were obtained from individuals 41 hours after wandering.

This experiment shows no effect of haemolymph

TABLE 4.4; Effect of haemolymph extract on secretion of ecdysone by PG from animals aged Eg;1.17.

Period of incubation (Mins)	Ecdysone secretion (ng) <sup>1</sup>	
	Control	Experimental
0 - 210	33.3 +/- 5.5	35 +/- 8.1
210 - 330	20.2 +/- 4.1	18.8 +/- 3.1
330 - 510	16.5 +/- 1.9	15.7 +/- 2.4

<sup>1</sup> Values given as means with standard errors.

None of the equivalent control and experimental secretions are significantly different ( $p \geq 0.2$ , t test). For further explanation of experimental conditions, see text.

extract on maintaining the high initial secretory rates of these glands. Preliminary experiments with glands of other ages, and various concentrations of haemolymph extract concentration also failed to enhance the secretory performance of the glands.

Other possible reasons for the observed decline in secretory activity are oxygen or substrate limitation. However, both these factors seem unlikely, as has been discussed above, and the results imply a requirement for constant stimulation of the glands by trophic factors such as PTTH or, possibly, nervous supply.

This system was considered adequate for use to study the effects of various substances on secretion of ecdysone by PG in vitro.

## CHAPTER 5

### EFFECTS OF EXOGENOUS ECDYSTEROIDS ON PROTHORACIC GLAND SECRETION IN VITRO.

Many developmental processes in insects are directly controlled by the changing concentrations of 20-HE (for reviews see Gilbert et al, 1980a; Riddiford, 1980c). In holometabola, such as Manduca, there are periods of rapid fluctuation of ecdysteroid titre which occur in a predictable manner (Bollenbacher et al, 1981). It is evident that these changes in ecdysteroid levels are precisely controlled. Indeed, adherence to a strict temporal sequence of fluctuation of both 20-HE and JH has been shown to be essential for the successful metamorphosis of a developing insect (Riddiford, 1976b, 1978).

The various factors which may contribute to control of fluctuation in ecdysteroid titre are discussed elsewhere in this thesis (see Chapter 2). However, it is apparent that an important consideration in the control of levels of 20-HE is the rate of synthesis of its immediate precursor, ecdysone. It has been demonstrated that PTTH is



able to cause an increase in secretory activity of PG, both in vivo (Gibbs and Riddiford, 1977) and in vitro (Bollenbacher et al, 1979). However, measurement of whole animal titres (Bollenbacher et al, 1981; Fig. 4.9) shows down-regulation of ecdysteroid titres also occurs, which correlate with reduced rates of ecdysone synthesis by PG (Bollenbacher et al, 1975; Fig. 4.8). It seems likely, therefore, that there is a regulating factor, causing reduction in ecdysone secretion at specific times during the developmental sequence. Such regulation could occur at a number of points in the ecdysteroid producing system, for example by regulation of PTH production by the brain, steroidogenesis by the PG, or ecdysone metabolism in tissues such as the fat body and midgut. Obvious candidates for such a role are ecdysteroids themselves, possibly regulating ecdysone synthesis by a feedback mechanism directly on the PG. The possibility that steroids may themselves exert an influence on steroidogenesis has been documented in several instances in work on vertebrate steroid hormones (see Gower, 1975 for review).

Several workers have suggested previously that ecdysone may act directly on the PG. Williams (1952b) showed that activation of PG of a brainless Cecropia pupa by brain implantation would result in activation of the glands of a second brainless animal joined in parabiosis. In this way, a chain of parabiosed, brainless animals would initiate development one after the other. If animals minus their PG as well as brainless were used, this response was abolished. This implied that the PG product, rather than the brain hormone was responsible for successive PG activation (ie. a positive feedback).

Siew and Gilbert (1971) showed an apparent stimulation of PG by 20-HE. Injections of 20-HE to diapausing silkmoth pupae led to dramatic increases in RNA synthesis (nuclear <sup>3</sup>H-uridine incorporation) in the gland cells. This implied activation of previously inactive PG by 20-HE. However, similar experiments with pupae which had initiated adult development showed a reduction of RNA synthesis after 20-HE injections, compared to control animals. The role of these two apparently opposite effects of 20-HE was discussed in terms of different effects depending on the existing

state of activity of the PG. Thus, activation of inactive glands ensured both glands secrete at the same time (as suggested by Williams, 1952b), whilst inhibition of active glands provides a control of titre of ecdysteroids by negative feedback.

Some of these results were incorporated into a hypothetical scheme of ecdysteroid titre regulation in Rhodnius by Steel (1975). He suggested 20-HE not only feeds back positively on the PG but, based on histological evidence, also stimulates further synthesis of brain hormone by neurosecretory cells in the brain.

Several other workers have reported apparent trophic effects of ecdysteroids on PG. Results obtained by incubating Mamestra wing discs with numbers of prothoracic glands (Agui and Fuykaya, 1973) suggested a synergistic effect of PG on each other, consistent with a positive feedback of the gland product. Similarly, Kimura and Kobayashi (1975) found ecdysone analogues were much more active in promoting development of brainless, diapausing Bombyx pupae when PG were present, than after their extirpation. Experiments on diapausing pupae of Manduca sexta led

Bradfield and Denlinger (1980) to conclude that exogenous 20-HE acted as a trophic agent on the PG, causing them to increase ecdysone synthesis which in turn accelerated the termination of diapause and completion of adult development.

It should be noted that all these studies involved experimental perturbations of intact animals, in which a variety of factors may act on the PG. The experiments described here were designed to investigate whether any role in modifying ecdysone synthesis by PG in vitro could be attributed to ecdysone or 20-HE.

### Methods

Ecdysteroids were measured spectrophotometrically (see Ch.3) and added to the culture medium at the required concentration. One of a pair of PG was incubated in ecdysteroid containing medium for between 1.5 and 2.5 hours, the other maintained in ecdysteroid free medium as a control. Glands were rinsed twice in fresh medium, and their secretory rates in normal medium assessed over 1.5 to 2 hours as described above (Chapter 4). A preliminary experiment showed that

100ng/ml of 20-HE had a repeatable effect on ecdysone secretory rates.

Effect of 20-HE on secretion of ecdysone by prothoracic glands in vitro.

Using this protocol, the effect of 20-HE on PG secretory activity was assessed at various times during the fifth larval instar (Table 5.1). From these results, it can be seen that a reduction in secretory rate of ecdysone is observed in the presence of 100ng/ml 20-HE. This effect is most pronounced for glands with high in vitro secretory rates. The reduction of ecdysone secretion by PG from Eg;1.17 animals was further investigated by incubating glands with various concentrations of 20-HE (Table 5.2). The percentage difference between the paired secretions ( $100 - (\text{experimental/control}) \%$ ) was calculated for the various concentrations of 20-HE (Fig. 5.1). As can be seen, no further reduction is achieved by incubating with concentrations of 20-HE greater than 100ng/ml (see Chapter 8 for discussion).

TABLE 5.1: Effect of 20-HE on secretion of ecdysone by prothoracic glands in vitro.

Age	N	Ecdysone secretion (ng/gland/hour)	
		Control	Experimental
L5;4.17 (Gate 2)	8	10.2 +/- 1.55 <sup>1</sup>	7.2 +/- 0.56 <sup>1</sup>
EG;0.17	6	9.1 +/- 1.06 <sup>2</sup>	7.6 +/- 0.9 <sup>2</sup>
EG;1.17	10	17.6 +/- 1.48 <sup>3</sup>	9.4 +/- 0.57 <sup>3</sup>

Results expressed as means +/- standard errors. <sup>1</sup> results significantly different (experimental < control),  $p \leq 0.005$ ; <sup>2</sup> results not significantly different,  $p \geq 0.05$ ; <sup>3</sup> results significantly different (experimental < control),  $p \leq 0.025$ . All by randomisation test for matched pairs (Siegel, 1956).

TABLE 5.2: Effect of various concentrations of 20-HE on secretion of ecdysone by EG;1.17 prothoracic glands in vitro.

N	Concentration of 20-HE (ng/ml)	Ecdysone secretion (ng/gland/hour)	
		Control	Experimental
6	1	16.5 +/- 1.18	16.9 +/- 1.75
6	10	13.2 +/- 1.14	12.1 +/- 1.06
10	100	17.6 +/- 1.48	9.4 +/- 0.57
6	500	15.1 +/- 1.02	10.3 +/- 1.55

Results expressed as means +/- standard errors.

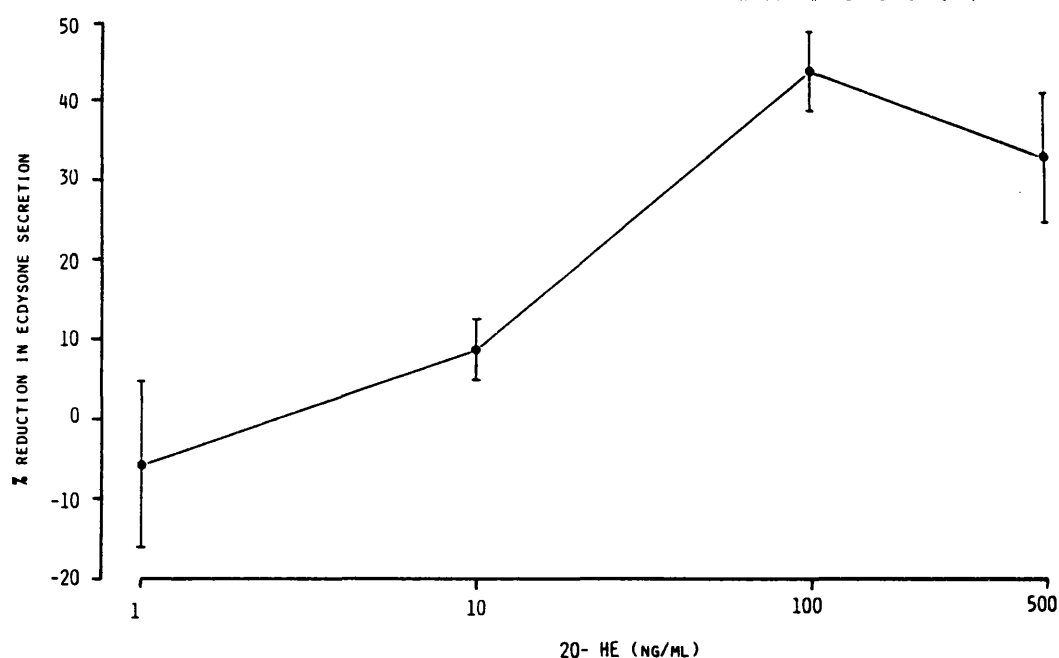


FIG. 5.1: REDUCTION IN ECDYSONE SECRETION BY PROTHORACIC GLANDS FROM ANIMALS AGED EG:1.17 INCUBATED WITH VARIOUS CONCENTRATIONS OF 20-HE. POINTS REPRESENT MEANS WITH STANDARD ERRORS OF AT LEAST 6 EXPERIMENTS.

Effect of ecdysone on secretion of ecdysone by prothoracic glands in vitro.

Among insect tissues, PG are unusual in being incapable of converting ecdysone to 20-HE (King, 1972; Bollenbacher et al, 1977). This implies that PG in vitro are probably exposed to a higher concentration of ecdysone (as distinct from 20-HE) than they would experience in vivo. Experiments were conducted to assess the effect of ecdysone on PG secretory rates in vitro.

Secretory rates of PG from Eg;1.17 animals were shown not to be modified in the presence of 100ng/ml ecdysone (Table 5.3). These results imply not only that the in vitro performance of PG is not modified by their own product, but also that the PG respond in a fundamentally different way to ecdysone and 20-HE. This observation is discussed in more detail in Chapter 8.



TABLE 5.3: Effect of 100ng/ml ecdysone on secretion of ecdysone by EG;1.17 prothoracic glands in vitro.

N	Ecdysone secretion (ng/gland/hour)	
	Control	Experimental
10	17.7 +/- 2.3	14.7 +/- 1.55

Results expressed as means +/- standard errors. Control and experimental secretions are not significantly different (randomisation test for matched pairs,  $p \geq 0.05$ ).

## CHAPTER 6

### EFFECT OF BRAIN EXTRACT ON PROTHORACIC GLAND

#### SECRETION IN VITRO.

PTTH has long been recognised as a regulator of the insect moult cycle, acting via the PG (see Chapter 1). More recently, it has been shown that PTTH acts directly on the PG to cause an increase in their rate of ecdysone secretion (Bollenbacher et al, 1979). During the larval - pupal transition in Manduca, there are known to be two separate periods when a factor from the head is required for successful moulting (Truman and Riddiford, 1974) and recent work has revealed PTTH peaks on both these occasions (Gilbert et al, 1981). However, not only does the internal milieu of the animal (ie. the environment of the PG) vary during the instar, but the glands themselves also vary (Chapter 2) and this may affect their ability to respond to PTTH.

The experiments described here are intended to investigate whether the ability of PG to respond to brain extracts with PTTH activity varies during the fifth instar.

## Methods

Brains were dissected from staged animals and cleaned of tracheoles and nerves, prior to washing in Manduca saline (Appendix B). They were pooled to give a concentration of 1 brain/20 $\mu$ l and disrupted with stainless steel needles. The resultant mixture was sonicated for a total of 30 seconds in three 10 second periods. The cell free solution was incubated at 100°C in a water bath for 5 minutes to destroy proteases. This extraction procedure was carried out as rapidly as possible to avoid loss of hormone activity.

The crude extract was centrifuged for 10 minutes in an Eppendorf bench-top centrifuge (type 5412, approximately 10Kg) to give a clear supernatant which was added directly to the culture medium at the required concentration. This brain extract had no activity in the RIA and so it was possible to measure the activity of glands directly in medium containing brain extract (rather than by preincubating them in the experimental medium and assaying subsequent secretory activity in fresh medium, as was done for the experiments in Chapter 5). Secretory rates

of PG were measured over 2.5 to 4.5 hours, using contralateral glands as controls.

Dose-response of activity of brain extract on prothoracic glands.

PG from donors aged EG;0.17 were chosen for this experiment, on the basis that they were competent to secrete ecdysone, but were not doing so at the maximum rate. Brain extract was prepared from animals aged L5;3.18, as preliminary experiments indicated these to be a good source of PTTH activity. Control experiments using extracts made from a variety of other CNS ganglia showed no change in PG secretory activity, presumably because these ganglia contained no PTTH. PG were incubated with brain extract concentrations between 0.01 and 2 brains/ml (Table 6.1; Fig. 6.1).

It is apparent from these results that a consistent increase of PG secretory activity is achieved with a concentration of brain extract of 0.5 brains/ml. It is unclear why a decrease in activation is observed with higher concentrations, but possibly some cytotoxic element in the extract

TABLE 6.1: Modification of secretory activity of prothoracic glands incubated with brain extracts of various concentrations.

Brain extract concentration (Brains/ml)	Ecdysone secretion <sup>1</sup>		
	Control <sup>2</sup>	Experimental <sup>2</sup>	Experimental /Control
0.01	6.68 +/- 0.26	6.62 +/- 0.76	0.98 +/- 0.08
0.1	6.34 +/- 0.57	8.09 +/- 0.56	1.32 +/- 0.15
0.5	6.89 +/- 0.9	13.23 +/- 1.44	1.96 +/- 0.12
1	7.88 +/- 1.11	11.28 +/- 1.15	1.48 +/- 0.13
2	6.44 +/- 0.27	6.1 +/- 0.79	0.94 +/- 0.09

<sup>1</sup> results expressed as means +/- standard errors of 5 experiments. <sup>2</sup> secretory rates expressed as ng/gland/hour.

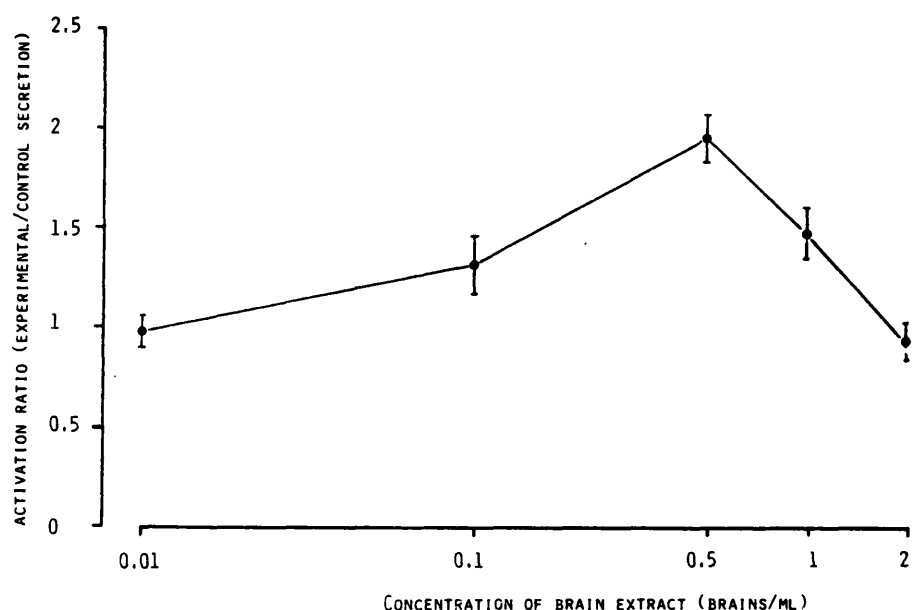


FIG. 6.1: IN VITRO ACTIVATION RATIOS OF PROTHORACIC GLANDS FROM ANIMALS AGED EG:0.17 EXPOSED TO VARIOUS CONCENTRATIONS OF BRAIN EXTRACT. POINTS REPRESENT MEANS WITH STANDARD ERRORS OF 5 EXPERIMENTS.

becomes sufficiently concentrated to limit gland activity in these conditions.

Subsequent incubations were performed with brain extract at a concentration of 0.5 brains/ml.

Responsiveness of prothoracic glands to PTTH at different stages of development.

Glands from donors of various ages between L5;0.17 and day 18 of pupal diapause (P 18, see Chapter 2) were incubated with the brain extract (Table 6.2). It can be seen that the greatest increase in secretory activity was obtained with PG from L5;2.17 animals. These glands have not yet been exposed to PTTH in vivo, and are ready to be activated. The only other stage at which PG are able to show an increase in secretory rate in response to brain extract is EG;0.17, just prior to the second in vivo increase in ecdysone production. It seems that PG respond to the brain extract only at those times when they are about to undergo an increase in their secretory activity in response to a trophic stimulus (PTTH) in vivo.

Gilbert, et al (1981) found PTTH present in the haemolymph from around L5;3.14 to L5;4.08 and

TABLE 6.2: Incubation of prothoracic glands from larvae of various ages with brain extract (0.5 brains/ml).

Age	N	Secretory rates (ng/gland/hour) <sup>1</sup>		
		Experimental	Control	Experimental/ Control
L5;1.17	4	4.2 +/- 0.25	4.2 +/- 0.29	1.02 +/- 0.05
L5;2.17	5	13.2 +/- 1.44	5.00 +/- 0.43	2.67 +/- 0.24
L5;3.18				
(gate 1)	4	10.9 +/- 1.49	10.75 +/- 0.87	1.03 +/- 0.05
EG;0.17	8	25.9 +/- 2.65	15.2 +/- 1.38	1.74 +/- 0.16
P18				
(diapause)	4	4.1 +/- 0.47	3.64 +/- 0.09	1.12 +/- 0.12

<sup>1</sup> results expressed as means +/- standard errors.

EG;1.12 to EG;1.18. In both cases where activation of PG by brain extract was achieved in this study (L5;2.17 and EG;0.17), the glands would have undergone in vivo activation within the next 24 hours. It seems likely that PG from animals earlier in the instar than L5;2.17 are incapable of responding to the brain extract, perhaps due to the steroid synthetic machinery of the gland cells not yet being complete, or a lack of PTH receptors in the cells. Glands from animals later in the instar which did not respond to the extract are probably already secreting at the maximum rate and therefore are unable to show an increase in ecdysone synthesis in response to the brain extract. It is interesting to note that PG from diapausing pupae are active at a low rate, but do not respond to the brain extract. These results are discussed further in Chapter 8.



## CHAPTER 7

### EFFECT OF JUVENILE HORMONE ON THE SECRETION OF ECDYSONE BY PROTHORACIC GLANDS IN VITRO.

The role of JH in the regulation of insect development has been discussed (see Chapter 1). Although some progress has been made in understanding the action of JH at the cellular level (eg. preventing the premature transcription of pupal or adult genes at a larval moult in Manduca, Riddiford, 1980a), there are many gaps in our knowledge of other aspects of JH activity, particularly concerning its interaction with ecdysone. Lezzi and Wyss (1976) reviewed examples of apparent antagonism between the actions of JH and ecdysone, but meaningful conclusions were prevented by the lack of accurate temporal measurements of JH and ecdysone levels. Several reports suggest JH exerts an effect on insect development by modification of the ecdysone titre, and some point to a direct regulation of PG activity by JH.

### Role of JH in termination of pupal diapause

Early work by Williams between 1946 and 1952 (Williams, 1952b) demonstrated that termination of pupal diapause in several Lepidoptera (notably Hyalophora cecropia) was due to the secretion of prothoracic gland hormone (ecdysone). In later work, the diapausing silkworm pupa was used extensively as an assay system for various insect developmental hormones. In particular, as prothoracic gland secretion was known to terminate diapause and to be stimulated by the brain hormone, treatments causing diapause termination in de-brained pupae could be assumed to be causing PG activation.

There have been several reports of the ability of corpora allata (CA) to stimulate moulting, and these were taken to imply a prothoracicotropic function of JH. Ichikawa and Nishiitsutsuji-Uwo (1959) reported that Philosamia pupae debrained two months previously could be stimulated to develop by CA implants. They interpreted these findings as evidence for storage of PTTH by the CA.

Williams (1959, 1961) performed similar work with the cecropia silkworm. He showed CA implants to elicit development in debrained pupae but not in isolated abdomens. The conclusion was that the action of the CA is directly on the PG. Williams concluded that the CA secrete a PTHH mimic, possibly identical with JH.

A JH extract was shown by Gilbert and Schneiderman (1959; Gilbert, 1962) to cause development of normal and debrained pupae, but not isolated abdomens, of several Saturniid (silkmoth) species to adult/pupal intermediates. However, contamination of the preparation by PTHH could not be ruled out. In the light of the more recent finding that the CA are, at least in Manduca, the release sites for PTHH as well as JH (Agui et al, 1980; Carrow et al, 1981), these three sets of results appear to be easily explained.

Although the chemical nature of JH remained unknown for a number of years, several mimics were discovered and Krishnakumaran and Schneiderman (1965) were able to demonstrate a positive correlation between the JH activity of several mimics (as measured by the Galleria bioassay, Schneiderman et al, 1965) and their ability to

activate dauer (debrained) pupae, presumably by switching on the pupal PG. They proposed the hypothesis that JH may maintain the PG at a low secretory rate during much of the instar, whilst PTH switches the glands to a higher level of secretion prior to apolysis. It was already known that the PG of Samia cynthia synthesise RNA throughout the fourth larval instar (Oberlander et al, 1965) and that ecdysone is maintained at a low but detectable level throughout the last larval instar in Calliphora (Shaaya and Karlson, 1965). Oberlander et al (1965) also reported that JH injections to Antheraea pupae caused increased RNA synthesis by the PG, implying a role for JH in PG activation.

This early work received support soon after JH had been purified (Röller et al, 1967; Meyer et al, 1968). In the course of investigation of the chemistry and biology of the molecule, its action in promoting adult development in brainless, diapausing silkworm pupae was confirmed (Dahm et al, 1968; Röller et al, 1969). This action of JH has recently been confirmed in cecropia (Bhaskaran et al, 1980) where it

causes diapause termination in brainless, allatectomised pupae.

Sehna et al (1981) also found that JH would initiate development in debrained pupae of Hyalophora cecropia and Celerio euphorbiae. However, whereas PTH (implanted brains) would give the same result when applied to isolated abdomens with PG implants, JH would not activate these transplanted glands. The implication is that JH acts not directly on the PG, but via a mechanism dependent upon the thorax, possibly upon a physical connection to the PG.

JH has also been shown capable of breaking diapause in adults of two non-Lepidopterous holometabolous insects (DeWilde and DeBoer, 1961; Bowers and Bickenstaffe, 1966). As no moult occurs, it seems unlikely in these instances that JH acts via the PG.

#### Role of JH in termination of larval diapause

Further information about the role of JH and the PG in insect diapause is provided by work on the larval diapause of certain Lepidoptera. As the larval diapause of Diatraea grandiosella is

thought to be maintained by a high JH titre (Yin and Chippendale, 1973; 1979), it seems unlikely that this acts to stimulate the PG. The ecdysiotropin (PTTH) producing system is thought, however, to be activated periodically during diapause, causing "stationary ecdyses" to a further larval stage. The frequency of these ecdyses is increased by JH application, but this effect is blocked by head ligation (Chippendale and Yin, 1976).

In Chilo suppressalis, diapause is also maintained and initiated by a high JH titre (Yagi and Fukaya, 1974). Here also, larval-larval ecdyses are undergone by the diapausing larvae and allatectomy, followed by JH application, showed JH to have an important role in initiating this. Similar JH dependent larval diapauses have been reported in a number of insects (Nair, 1974; Yagi, 1976). The exact target for JH in this apparent stimulation of the moult controlling system is unclear.

Evidence from Masner et al (1976), Mitsui and Riddiford (1978) and Sehnal et al (1981) indicates that JH lowers the threshold requirement of the epidermis for ecdysteroids, thus allowing

cuticle synthesis at lower ecdysteroid titres. This may well explain the occurrence of stationary ecdyses during larval diapause, on the basis of low level activity of the PG and high JH titres.

#### Antagonistic interactions between JH and ecdysteroids

The findings reviewed above all suggest a synergistic interaction between JHs and ecdysteroids, either direct (by action at the level of the target tissues) or indirect (mediated by JH acting to increase the ecdysteroid titre). However, there is also evidence that JHs can interact antagonistically with ecdysteroids. In normal development of Manduca, Nijhout and Williams (1974a; b) showed that a train of events is initiated once larvae pass a critical weight threshold, culminating in the pupal moult. The first mediator of this response is a decline in the JH titre, which appears to allow the prothoracicotropic system to be activated. It has since been shown that a specific JH esterase, which becomes detectable in the haemolymph at about day 2 of the last larval instar, and peaks

at the onset of the pupal stage, acts as a fail-safe mechanism to ensure the JH titre declines at the correct time (Vince and Gilbert, 1977). It seems likely, therefore, that in this instance JH acts upon the PTTH secreting cells in the brain, rather than directly on the PG. There is evidence to suggest a similar role for JH in control of the onset of the larval-pupal transformation in Galleria (Ciemior et al, 1977; Hwang-Hsu et al, 1979) and Spodoptera (Cymborowski and Stolarz, 1979).

A similar response of the PTTH producing system to JH appears to occur during the prepupal diapause of Monema flavescens (Takeda, 1978). This diapause appears to be maintained by high levels of JH in the haemolymph and further development is not initiated until the titre falls. Histological evidence suggests that the role of JH in diapause maintenance is mediated by an inhibitory effect on the release of neurosecretory material from the B cells of the pars intercebralis. It is uncertain how these cells relate to the prothoracicotropes (Gilbert et al, 1981) of Manduca, as the prothoracicotrophic neurosecretory material which



they produce is secreted directly into the haemolymph (Takeda, 1972; 1976b) and taken up (Takeda, 1977) and transported by haemocytes to the prothoracic glands (Takeda, 1976a).

Evidence for antagonistic interactions between JH and ecdysteroid systems is not restricted to Lepidopterous larvae. For instance, in the cockroach Blatella germanica, treatment with JH or a synthetic analogue (6,7- epoxyl-1-(p-ethylphenoxy)-3,7-dimethyl 1-2-octene) has been shown to inhibit the normal rise in ecdysteroid titre prior to moulting (Masner et al, 1976). The target for JH in producing this effect is not known.

An inhibitory effect of JH on ecdysteroid mediated events has been reported in Blatella (Masner et al, 1976) and in Dermestes by Delbeque and Slama (1980) who concluded the effect to be more probably mediated by modification of the peripheral response rather than by affecting the central neuro-endocrine system.

### Role of JH in Manduca diapause

Investigation into the control of the pupal diapause of Manduca has revealed major differences between this species and the larval diapause described in Chilo, Diatraea and Monema (Bradfield and Denlinger, 1980). It appears that JH has no role in maintenance of this diapause (Chapter 2), being undetectable in the haemolymph of diapausing pupae by the black larval Manduca bioassay (which has a lower limit of detection of 0.04 ng C-18 JH (JH1) equivalents/ml haemolymph, Fain and Riddiford, 1975) throughout the diapause period. Allatectomy was found to have no effect on diapause termination, which is puzzling in view of the CA being known to secrete both JH and PTH in Manduca. Although JH treatment was able to terminate diapause, a non-physiological dose of around 2.5 µg/g whole animal weight of the JH analogue ZR515 (which was calculated to give an immediate haemolymph titre of 5 µg/ml JH activity) was required, implying that JH is not normally responsible for PG activation at this time.

### Stage specific effects of JH

Work on the effects of JH during the larval-pupal transformation of Manduca has revealed that JH treatments delay the onset of wandering, but if applied after this, accelerate pupation (Safranek et al, 1980). Whilst the brain was found unnecessary in the mediation of this response, the authors could not identify the exact site of JH action, but suggested it to be either the synthesis of ecdysone by the PG, rate of 20-hydroxylation of ecdysone, rate of inactivation of ecdysteroids or modification of the cellular response to ecdysteroids.

Recent experiments by Hiruma and co-workers on larvae and pupae of Mamestra brassicae provide evidence for the possibility of JH activating the PG in a similar stage-specific manner (Hiruma et al, 1978a). They showed that larvae deprived of their brains would respond to treatment with a JH analogue (JHA, in this case ZR 515) by pupating. Hence, head ligated wandering larvae (day 5 after the last larval moult) and isolated day 5 abdomens with implanted day 5 PG pupated after JHA application. Day 3 larval

abdomens (pre-wandering) with day 5 PG implanted formed larval-pupal intermediates on JHA application. Isolated abdomens with no implants failed to respond to application of the same synthetic JH analogue. In experiments with pupae, non-diapausing animals showed an acceleration of the onset of adult development after JHA treatment, and dauer (brainless) pupae broke their enforced diapause, as did normal pupae, in response to JHA.

The response to JHA was different to that to PTTH, however, as brain implants caused pupation of neck-ligated pre-wandering larvae, and also day 5 isolated abdomens with pre-wandering PG implants, neither of which preparations responded to JH.

Further work on this system has shown that the rise in ecdysone titre (measured by RIA) following JH application only occurs after the wandering stage (Hiruma, 1982; Hiruma and Agui, 1982). The JHA doses used in these experiments were well within physiological limits, and doses as low as 1 to 5ng per whole animal could elicit the pupation response.

Similar observations have been made on

Spodoptera by Cymborowski and Stolarz (1979). These authors hypothesise that if JH does not act directly on the PG, it could act by inhibiting the release of stored PTH from thoracic ganglia.

The conclusion to be drawn from all these experiments is that JH acts on wandering larvae and pupae (but not feeding larvae) to promote ecdysteroid dependent development. The results are consistent with the hypothesis that JH activates the PG, but are not sufficient to prove it, since the problem remains that using whole animal preparations precludes proof of a direct effect on the PG. Other explanations of the results remain possible. For example, applied JHA may either cause release of "stored" ecdysone from inactive conjugates, increase the rate of 20-hydroxylation of ecdysone, or enhance the sensitivity of the epidermis to 20-HE. These matters are considered further in Chapter 8.

The experiments described here were designed to investigate whether JH can directly influence the secretion of ecdysone by the PG in Manduca, thus at least potentially having a role in control of the ecdysone titre.

## Methods

Prothoracic glands were incubated as described above (Chapter 4), in the presence of JH (5µg/ml JH1, Calbiochem). Contralateral glands were incubated in normal medium as controls. As JH was not active in the RIA, it was possible to assay directly the amounts of ecdysone secreted by PG into JH containing medium.

### Effect of JH on secretion of ecdysone by prothoracic glands in vitro

In a preliminary experiment, glands from L5;3.17 and EG;1.17 individuals were exposed to JH for 1 hour. Their secretory rate was assessed during the following 3 to 4 hours. Control and experimental secretions were not significantly different (Table 7.1). Subsequent experiments were conducted by exposing the experimental gland to JH for the duration of the experiment, usually 5 to 6 hours.

To determine whether JH had a demonstrable effect on the in vitro secretion of ecdysone by PG at different developmental stages, PG from

TABLE 7.1: Effect of exposure to juvenile hormone for 1 hour on rates of ecdysone secretion by prothoracic glands in vitro.

Age	N	Secretory rates (ng/gland/hour)		
		Experimental <sup>1</sup>	Control	Activation ratio
L5;3.17				
(gate 1)	4	8.1 +/- 1.76	10.5 +/- 1.59	0.79 +/- 0.14
EG;0.17	7	9.88 +/- 1.02	10.16 +/- 0.56	0.99 +/- 0.12

Secretory rates expressed as means +/- standard errors. <sup>1</sup> the experimental and control secretions are not significantly different ( $p \geq 0.05$ ) by randomisation test for matched pairs. For experimental protocol, see text.

animals of various ages were incubated with (experimental) or without (contralateral control) JH. None of these experiments showed a significant difference between control and experimental secretion (Table 7.2).

#### Experiments on head ligated animals

The above experiments demonstrate that even at the relatively high concentration of 5 µg/ml, JH does not have an effect on PG secretion of ecdysone in vitro, under the conditions used. As PG activation had been achieved under similar conditions using brain extracts, it seems unlikely that JH can alter the secretory rates of PG extirpated at the times shown in Table 7.2. However, this does not preclude an action of JH in modifying PG secretion in vivo. It may be that the PG had already been exposed to JH before extirpation for the experiment. The haemolymph titre of JH in Manduca has been shown to decrease dramatically before the first small peak of ecdysteroid prior to wandering (Riddiford, 1980a). It seems unlikely, therefore, that this first round of PG activation is caused by JH.



TABLE 7.2: Effect of exposure to juvenile hormone on rates of ecdysone secretion by prothoracic glands in vitro.

Age	N	Secretory rates (ng/gland/hour)		
		Experimental <sup>1</sup>	Control <sup>1</sup>	Activation Ratio
L5;2.19	5	6.54 +/- 0.66	6.54 +/- 0.32	1.03 +/- 0.05
L5;3.19	9	9.69 +/- 0.65	9.37 +/- 0.75	1.05 +/- 0.05
EG;0.18	4	11.24 +/- 0.6	10.24 +/- 0.36	1.1 +/- 0.08
EG;1.18	5	21.1 +/- 2.29	19.79 +/- 1.04	1.07 +/- 0.11

Glands were incubated with or without JH for 4 to 5 hours, and their secretion of ecdysone assayed. Secretory rates given as means with standard errors. <sup>1</sup> experimental and control secretions not significantly different (randomisation test for matched pairs,  $p \geq 0.05$ ).

However, the JH titre rises again at almost exactly the same time as the edysteroid titre for the post-wandering peak. It was decided to investigate the relationship (if any) between JH and the acceleration of ecdysone synthesis by PG (see Chapter 4) at this time.

Neck ligation or brain removal from Manduca larvae immediately after entry to the wandering stage increases the time to pupation from four days to more than seven days (Truman and Riddiford, 1974; Safranek and Williams, 1980). This delay has been presumed to be caused by the removal of the source of PTTH. However, these experimental manipulations could also have had their effect by reducing the JH titre. These larvae can be "rescued" (ie. returned to a developmental fate more closely resembling the normal situation) by the application of JH analogues (JHA) immediately after ligation (Safranek et al, 1980). It remains unclear whether the ability of JHA to accelerate development in the ligatured larvae is due to an action on the PG, causing the required increase in ecdysone secretion, or on the epidermis, making

the latter more responsive to the low levels of ecdysteroid available.

The experiments described here were designed to test directly the effect of JH on PG from fifth instar larvae head ligated immediately after entry to the wandering stage. Animals were neck ligated 4 hours after lights on, on the day immediately after wandering. These animals typically formed tanned pupal cuticles in 10 to 12 days, though there was some mortality prior to this. All animals observed (>30) survived for more than 7 days after ligation. Ligatures were applied with dental floss after animals had been anesthetised by brief (about 5 minutes) immersion in tap water. After ligation, the anterior portion was destroyed by removing the front of the head capsule.

Secretion of ecdysone by prothoracic glands from head ligated animals *in vitro*.

Initially, the *in vitro* rate of ecdysone secretion for PG from head ligated animals was measured (Table 7.3). The secretory rates were significantly below those found in normal animals (Table 4.3, Chapter 4) and exhibited considerable

TABLE 7.3: In vitro secretory rates of prothoracic glands from larvae head ligated after entering the wandering stage.

Age	N	Ecdysone secretion (ng/gland/hour)
EG;1.17	8	5.13 +/- 0.53
EG;2.17	8	5.92 +/- 0.82

Secretory rates given as means with standard errors.

variation between individuals. However, as observed for PG from normal animals, left and right glands secreted at the same rates (left glands secreted at  $101 \pm 3.1\%$  (mean and standard error) of the rate of right glands,  $n=8$  pairs) allowing assessment of the effect of JH on secretory rates by using contralateral controls as described above.

Effect of JH on *in vitro* secretory rates of prothoracic glands from head ligated larvae.

PG from animals head ligated immediately after wandering were incubated with and without JH (Table 7.4). JH appeared to be incapable of enhancing the *in vitro* secretion of ecdysone by these glands. This implies that the effect of reducing the delay in pupation after head ligation is not due to direct action of JH on the PG. These results are discussed further in Chapter 8.

TABLE 7.4: Effect of exposure to juvenile hormone on rates of ecdysone secretion by prothoracic glands from head ligated animals in vitro.

Age	N	Ecdysone secretion (ng/gland/hour)		Activation ratio
		Experimental <sup>1</sup>	Control <sup>1</sup>	
EG;1.17	8	9.46 +/- 1.8	9.66 +/- 1.97	1 +/- 0.03
EG;2.17	8	12.24 +/- 2	11.86 +/- 2.25	1.1 +/- 0.08

Secretions given as means with standard errors.

<sup>1</sup> Experimental and control secretions not significantly different ( $p \geq 0.05$ , randomisation test for matched pairs).

## CHAPTER 8

### GENERAL DISCUSSION

The aim of this project was to investigate some of the factors which may control the rate of secretion of ecdysone by the PG during the larval-pupal transformation of Manduca. The underlying problem is that of how the fluctuating levels of ecdysteroids, in particular 20-HE, are controlled in order to yield the temporal pattern of ecdysteroid titres vital to successful metamorphosis.

To interpret the results, it must first be considered whether the PG in vitro are likely to respond to exogenous stimuli in a similar manner to the response they would give in vivo. Some measure of the suitability of the in vitro technique can be obtained by comparing the secretory performance of PG in vitro to that in vivo. This latter property can not be measured directly, due to the complex nature of the metabolism of ecdysone secreted in vivo (Koolman, 1980b), but an estimate can be obtained by comparing whole animal ecdysteroid

titres with the secretory rate of glands in vitro, multiplied by the time available to produce the in vivo titre.

#### Rate of ecdysone secretion by PG in vitro

Incubations of PG from animals early in the fifth instar resulted in approximately linear relationships between time and amount of ecdysone secreted (Figs. 4.1, 4.2, 4.3). These results probably reflect constant, low level activity of the PG, as has previously been reported (Bollenbacher et al, 1975; Nijhout, 1976). Incubation of PG from animals later in the instar (during periods when the whole animal ecdysteroid titre is rising rapidly) resulted in non-linear secretion of ecdysone with respect to time (Figs. 4.4, 4.5, 4.6, 4.7). Attempts to lengthen the period for which these PG would secrete at the maximum in vitro rate, by modifying the in vitro environment, were unsuccessful (Chapter 4). It can be concluded either that the declining secretory rates of the PG observed in vitro are already programmed, at the time of the glands' extirpation, or that some aspect of their in



vivo environment is absent in the incubations. The former explanation seems unlikely. Possibilities for the latter include substrate limitation, inadequate culture conditions or lack of a constant trophic stimulus which is present in vivo.

Substrate limitation has previously been considered a possible reason for sub-optimal secretory performance of PG in long term cultures (see Chapter 1). However, the exact nature of the substrate utilised by the PG for ecdysone biosynthesis is uncertain. Whilst several studies have used labelled cholesterol as a precursor for ecdysone synthesis in vitro, low yields of labelled ecdysteroids were obtained (Willig et al, 1971; King, 1972; Bollenbacher et al, 1976) and more recent work has implied that cholesterol may not be the substrate normally used by the glands (see Gilbert et al, 1977 for review). It is to be anticipated that the sterol precursor of ecdysone would be present in insect haemolymph. In the present work supplements of haemolymph had no effect in modifying PG secretory activity. This provides good evidence to suggest substrate deficiency was not a limiting factor in the

synthesis of ecdysone by the PG in the experiments reported here.

The culture conditions used allowed constant, low level secretion of ecdysone for up to 24 hours<sup>(results not shown)</sup>. Microscopic examination of the PG after this time showed the glands to be of similar appearance to when first isolated. Changing the culture medium at different intervals had no effect on the rate of ecdysone secretion (results not shown). Hence, the culture conditions were probably adequate to maintain the in vivo secretion of ecdysone by the PG.

PG in vivo probably receive both humoral and nervous stimuli. Whilst the humoral stimuli can be mimicked in vitro, the nervous ones cannot. Several lines of evidence suggest these nervous connections may be important in maintaining the normal function of the PG (see Chapter 1). It may be the case that the removal of the nervous connections is a major factor contributing to the inability of the glands to maintain high in vitro secretory rates. The role of humoral stimuli in modifying PG secretory rates is discussed below.

Comparison of in vitro ecdysone secretion  
with in vivo titres

Due to the considerations outlined above, the initial secretory rate of isolated PG seems most likely to approximate to the true in vivo performance. As PG do not store ecdysone, it is unlikely that a transient increase in release occurs after extirpation of the PG. Hence, the initial rate at various times during the instar was plotted against the age of the animal (Fig. 4.8). The area under the curve for this graph represents an approximation of the amount of ecdysone secreted by PG in vivo. If losses to the system are ignored (ie. ecdysteroid metabolism and excretion), then a cumulative representation of this data gives a graph showing the total amount of ecdysone secreted in vivo prior to a given developmental stage. This data can be compared to whole animal titres for free ecdysteroid obtained by RIA of whole animal extracts (Fig. 8.1, Table 8.1).

Unfortunately, it is difficult to compare the two sets of data in Fig. 8.1 objectively as there is insufficient data available concerning the fate

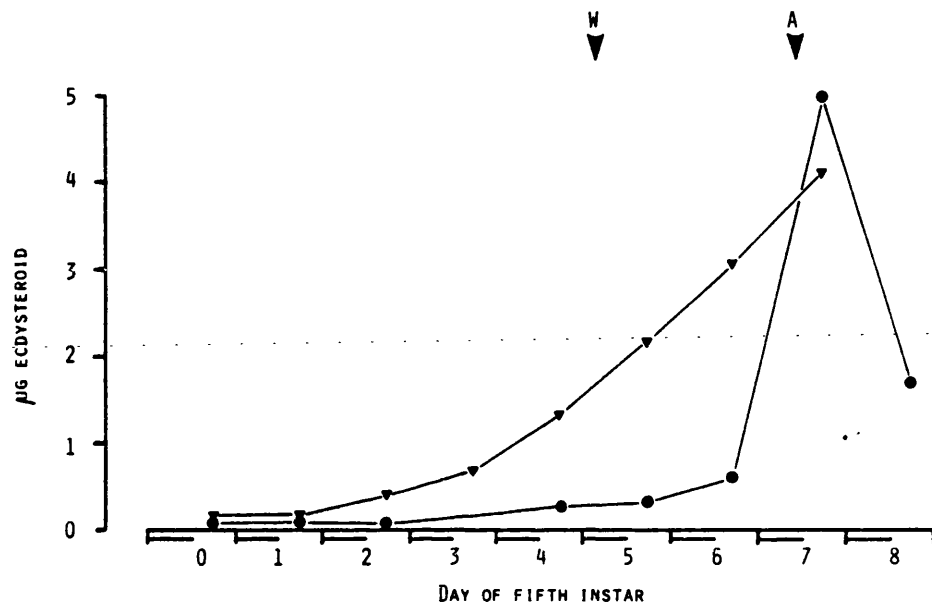


FIG. 8.1: COMPARISON OF CUMULATIVE IN VITRO ECDYSONE SECRETION BY PROTHORACIC GLANDS (▼) WITH WHOLE ANIMAL ECDYSTEROID TITERS (●) FOR GATE 2 ANIMALS. SEE TEXT FOR DETAILS. W REPRESENTS TIME OF ENTRY TO WANDERING STAGE, A REPRESENTS TIME OF FIFTH INSTAR APOLYSIS.

TABLE 8.1: Cumulative ecdysone secretion by PG in vivo,  
assuming constant secretion at initial in vitro rate.

Age	Cumulated ecdysone secretion (ng/gland pair)	
	Gate 1	Gate 2
L5;1.17	153.2	153.2
L5;2.17	414.4	414.4
L5;3.17	871.5	699.6
L5;4.17	-	1324.6
Eg;0.17	1562.7	2183.8
Eg;1.17	2455.9	3076.6
Eg;2.17	3487.9	4108.6

Glands were assumed to secrete constantly at the measured in vitro rate for the period up to the succeeding measurement. Totals represent cumulated amount of ecdysone secreted by a pair of PG from L5;0.17 up to the developmental stage indicated.

of ecdysone secreted in vivo. Whilst the PG in vitro secrete at a rate which would be capable of providing an ecdysteroid titre within the order of magnitude of that measured in vivo, there are a number of unknown parameters, such as the rate of metabolism of ecdysteroids at different times during the instar and the efficiency of RIA on whole animal extracts (see Chapter 3) which make direct comparison inappropriate.

However, it can be seen that the agreement between the two curves is not particularly good (Fig. 8.1), and so it is unlikely that regulation of in vivo titres is accomplished solely by variation of PG secretory rate coupled with ecdysteroid metabolism proceeding at a constant rate. Hence, it is to be expected that the rate of ecdysteroid metabolism varies during the fifth instar in Manduca, and indeed there is evidence to suggest this may be so (see below).

## Modification of *in vitro* secretory rates

### Ecdysteroids

Three types of humoral stimuli were used in an attempt to modify the *in vitro* rate of secretion of ecdysone by PG. The first of these, ecdysteroids, produced results indicating that 20-HE, at a level within the measured limits of haemolymph titre (Bollenbacher et al, 1981) could modify PG secretion, having a direct feedback inhibitory effect on the glands (Fig. 5.1). This is in agreement with a previous suggestion of an inhibitory effect of 20-HE on active PG (Siew and Gilbert, 1971).

Interestingly, ecdysone had no effect on the secretory rates of cultured PG. This implies that the *in vivo* secretory rate is affected not by the amount of ecdysone already secreted, but only by the 20-HE titre, itself dependent upon ecdysone monooxygenase activity. The implications of this for the control of PG activity *in vivo* are discussed below. A further implication of this finding is that the ecdysone secreted by the PG *in vitro* would have no effect on the subsequent secretory activity of the gland, ie.

the fall off in the secretory rates of some PG which was observed (see above) was not due to the action of the secretory product on the gland.

#### Brain extract

The brain extract used in experiments described in Chapter 6 was also successful in modifying PG secretory rates. However, quite high doses of extract (0.5 brains/ml) were required to give a 96% activation of EG;0.17 glands and higher doses resulted in reduction of this activation ratio. Glands from younger animals (L5;2.17) showed a greater proportional response to the brain extract, so it is possible that these would have shown activation with lower concentrations of the extract.

Bollenbacher et al (1979) were able to achieve activation of day 0 pupal Manduca PG with as little as 0.1 brain equivalents from day 6 larval brains. However, as their cultures used a volume of only 0.025mls, the concentration of brain extract to which the PG were exposed was, in fact, 4 brain equivalents/ml.

It is unclear why a reduction in activation ratio of the PG was achieved by higher



concentrations of brain extract. One possibility is that toxic substances present in the extract become inhibitory to ecdysone secretion at these levels. An alternative explanation is that the brain contains a substance which specifically inhibits the production of ecdysone by the PG. The existence of such a PG inhibiting factor has been reported previously in a different species (see Chapter 1) and so it is not impossible that a similar factor may be present in Manduca. However, the evidence provided by the experiments reported here is insufficient to postulate the existence of such a factor. In any case, it would appear that in the experiments of Bollenbacher et al (1979), no such inhibition of PG function by high doses of brain extract was seen.

#### Juvenile hormone

The third humoral factor used in attempts to modify the PG secretory rates was JH (Chapter 7). No effect of JH could be demonstrated either on PG from normal animals of various ages (Table 7.2) or on animals head ligated just after wandering. Head ligation at this time removes the sources of both PTH and JH from the PG. The delay in pupation and

reduction in secretory rates of PG in vitro (compare Tables 7.3 and 4.3) implies that a factor from the head is required to maintain the high rate of secretion of the PG. Unfortunately, the effect of brain extract on these PG was not tested, but results shown in Table 7.4 indicate that JH was unsuccessful in activating PG from these head ligated animals.

As modification of PG secretion was obtained with both 20-HE and brain extracts, and a relatively high dose of JH (5µg/ml) was used in the experiments, it seems unlikely that JH acts directly on the PG to modify their secretory activity in vivo. These results are in agreement with those of Sehna et al (1981) who identified several points at which JH apparently regulated ecdysone production, none of which involved a direct effect on the PG (see Chapter 7).

As discussed in Chapter 7, there are numerous reports of JH apparently modifying the in vivo secretion of ecdysone by PG. However, in each case, whole animal preparations have been used, which precludes demonstration of a direct effect of JH on the PG.

Once Manduca epidermis has been exposed to 20-HE in the absence of JH it becomes pupally committed (Riddiford, 1976b). This means that the epidermis is no longer able to synthesize larval cuticle and, upon the next exposure to 20-HE, begins to synthesize pupal cuticle. Whilst JH is present during this second rise in ecdysone titre prior to pupal cuticle synthesis, it is not needed for production of the pupal cuticle. One role of JH at this time appears to be to prevent premature activation of adult genes (Kiguchi and Riddiford, 1978). It is possible that JH may also function to modify ecdysteroid metabolism, resulting in an increase in 20-HE available to the tissues. Such a role for JH could explain the apparent reduction of threshold for ecdysteroids required for moulting in JH treated animals (Mitsui and Riddiford, 1978; Sehnal et al, 1981). Alternatively, these results may reflect an action of JH in increasing the sensitivity per se of the epidermis to ecdysteroids.

### Relationship between PG protein content and ecdysone secretion

PG isolated from Manduca larvae at various times during the fifth instar vary remarkably in their ecdysone secretory rates (Fig. 4.8). However, they do secrete detectable amounts of ecdysone at all times. The protein content of the glands also changes, in a manner not totally dissimilar to the fluctuation in ecdysone secretory rates (Fig. 2.3).

From these results, the rate of synthesis of ecdysone per mass of protein can be calculated (Table 8.2). It can be seen that steroid synthesis is not directly related to the protein content of the PG. As the experiment detailed in Table 2.4 shows that the rise in protein content of the glands is not due to PTTH secretion, but occurs before it, it seems likely that the glands synthesize new protein prior to PTTH stimulation, but this new synthetic capacity is not realised until activation by PTTH. The possibility that the rapid rise in protein content of PG is itself under humoral control requires further investigation. The activation factor present in

TABLE 8.2: Specific rate of ecdysone synthesis by prothoracic glands from larvae of various ages.

Age	Protein content ( $\mu\text{g/gland}$ ) <sup>1</sup>	Ecdysone secretion ( $\text{ng/hour}$ ) <sup>2</sup>	Specific rate of ecdysone synthesis ( $\text{mg/hour/g}$ )
L5;1.17	7.0	4.94	0.71
L5;2.17	9.7	5.94	0.61
L5;4.17			
(gate 2) 21		20.1	0.96
Eg;0.17	21.8	15.7	0.72

<sup>2</sup> Data from Table 2.3, <sup>1</sup> Data from Fig. 4.8.

the brain of Periplaneta has been shown to cause RNA synthesis but not ecdysone synthesis in PG (Gersch, 1980). It is possible that a similar factor is present in Manduca and accounts for the sudden onset of protein synthesis prior to increased steroidogenesis.

#### Control of in vivo PG activity

There are four major changes in the secretory activity of the PG during the larval-pupal transition in Manduca, three of which are documented in Fig. 4.8. These are the rapid rise in secretory rate prior to wandering, the reduction in secretory rate at EG;0.17 (only detected in gate 2 animals), the rise before pupal cuticle synthesis and finally, not shown in the figure, the cessation of ecdysone secretion. It is uncertain when this last change occurs. Bollenbacher et al (1979) report day 0 pupal glands to be actively secreting ecdysone at above minimal levels in non-diapausing animals, and so the PG may not be switched off between pupal ecdysis and the onset of adult development in long

day conditions. It is likely that there is a gradual decrease in PG activity after apolysis to the pharate pupal stage (Bollenbacher et al, 1975).

There is little doubt that PTH is responsible for the first rise in PG secretion. This has been demonstrated by ligation experiments (Truman and Riddiford, 1974) and direct activation of PG by brain extracts (Chapter 6). This initial rise in secretion of ecdysone is short lived, however, and the rate of secretion decreases again within 24 hours in gate 2 animals at least (Fig 4.8, B). Such a decrease was not detected for gate 1 animals which, at L5;3.17, had a lower in vitro secretory rate than gate 2 animals 1 day later. This is probably due to gate 1 animals releasing PTH later in the day than the gate 2 animals, having become competent to release PTH during, rather than prior to the permissive period. Hence, PG from gate 1 animals at L5;3.17 have been exposed to PTH for less time than gate 2 animals at L5;4.17.

There are several possible reasons for this reduction in secretion. It may be that the PG are only partly activated by the first release of PTH

and so the increase in ecdysone secretion is short lived. Alternatively, the PG may respond to increasing levels of 20-HE by reducing their rate of ecdysone secretion. The experiments detailed in Chapter 5 seem to support this idea. A third explanation is that the in vivo environment of the PG is not adequate to support prolonged ecdysone secretion. The possibility of the PTH released during the first and second pulses being different hormone species is discussed below.

One difference between the environments experienced by the PG during the first and second rounds of secretion is that JH is absent during the first and present during the second (see Chapter 1, Fig. 1.2). It is possible that JH present in the haemolymph during the major period of ecdysone production acts in some way to maintain the high secretory ability of the gland at this time. Also, the PG, like all the other tissues, have been exposed to ecdysone in the absence of JH for the first time in their development. This may modify the gland cells so that they respond to the second trophic stimulus in a different manner to that in which they respond to the first.



Should JH have a role in modifying the secretory ability of the PG, it is unlikely that it acts alone in this. All attempts to modify the in vitro secretion of ecdysone with JH failed (Chapter 7) and so it is unlikely that the second rise in ecdysone secretion in vivo is solely due to the temporally correlated increase in JH levels. More likely, the second signal from the head required for pupal development represents a second release of PTTH. Not only have ligation experiments revealed the head to be active at this time (Truman and Riddiford, 1974) but also, brain extracts were able to activate EG;0.17 PG in vitro (Chapter 6).

After the second pulse of ecdysone secretion, secretory activity of the PG again declines. It is uncertain whether a specific stimulus actually switches the glands off. If, as is likely, the glands require constant trophic stimulation for continued secretion, then elimination of the trophic factor from the haemolymph would result in gradual switching off of ecdysone secretion.

Preliminary evidence (Gilbert et al, 1981) has revealed the possibility that PTTH exists in two forms in the pupal brain of Manduca. These

two forms have different molecular sizes, and may be distinguished as "small" and "large" PTH. Gilbert et al speculate that the smaller of these is released during the first PTH pulse, and the larger during the second. If this is indeed the case, then an elegant hypothesis for the differential control of the two bursts of PG activity can be formulated on the basis of different responses of the glands to each form of PTH, or different half lives of the two forms in the haemolymph. However, as noted above, a difference in response of the gland cells to the two PTH releases can adequately explain the different secretory characteristics observed.

The response of the PG to 20-HE (Chapter 5) also provides a possible explanation for the decrease in secretory rates after apolysis. This feedback effect of 20-HE is probably more important after the second peak of ecdysteroids than after the first. The haemolymph ecdysteroid titre rises to about 80ng/ml after the first pulse of ecdysone secretion, but as the ratio of ecdysone to 20-HE is relatively high at 1:1.1 (Bollenbacher et al, 1981), the titre of 20-HE is only about 40 ng/ml. Ecdysone was ineffective

in modifying ecdysone secretory rates, and 100ng/ml of 20-HE was required to produce a significant reduction in PG activity (Fig. 5.1). Hence, the level of 20-HE in the haemolymph probably does not limit PG activity during the first period of ecdysone secretion. During the second peak of ecdysteroids, the titre reaches 1.5µg/ml, and the ecdysone to 20-HE ratio is 1:5.4. This level of 20-HE is adequate to cause an inhibition of PG activity, as observed in vitro.

To summarise, both first and second increases of the ecdysone secretory rate are probably due to secretion of PTTH by the brain. The difference in the persistence of the effect of PTTH in each case may be due to differences in the nature or duration of the trophic stimulus, or differences in the environment or status of the PG in each case. The feedback inhibition of ecdysone secretion by 20-HE probably regulates PG activity only during the second period of ecdysone production.

### Control of 20-HE titre

Numerous reports suggest 20-HE to be the chemical species involved in modifying gene expression at the target tissue during ecdysteroid control of insect development (see Chapter 1). Regulation of the titre of 20-HE therefore controls insect moulting. Ecdysone production by the PG is only one of numerous factors which affect this titre. The metabolism of steroids in insects is complex (see Gilbert et al, 1980b for review) and several different processes contribute to the rate of formation and inactivation of 20-HE. As discussed above, whilst the rate of production of ecdysone by the PG fluctuates during the instar, these fluctuations are insufficient to account for the measured variation in free ecdysteroid titre, if other aspects of ecdysone metabolism were to remain constant. There are, however, several reports of fluctuations in enzyme activity important in ecdysone metabolism, though in general more data is required to produce a comprehensive account.

### Rate of 20-hydroxylation of ecdysone

Production of 20-HE depends upon the presence of the enzyme ecdysone monooxygenase, and its substrate, ecdysone. The variable activity of the PG provides temporal regulation of substrate availability, but it appears that the enzyme activity may also vary. During Bombyx development, the rate of conversion of ecdysone to 20-HE increases prior to pupation, whilst further metabolism of 20-HE decreases (Moriyama et al, 1970). Koolman (1980b) has reported that in Calliphora the rate of 20-hydroxylation of ecdysone peaks towards the end of the third (last larval) instar. The rate of conversion of ecdysone to 20-HE also varies considerably during locust development (Hoffman et al, 1974; Feyereisen et al, 1976). Koolman et al (1975) report maximum ecdysone monooxygenase activity coincides with the major peak of ecdysteroids, and Feyereisen and Hoffman (1977) have provided evidence suggesting ecdysone monooxygenase activity is induced by high titres of ecdysone in Locusta.

These reports provide ample evidence that ecdysone monooxygenase is indeed developmentally

regulated and this is likely to be a feature common to many insect systems.

#### Metabolism of 20-HE

Whilst there are several possible routes for 20-HE inactivation (see Chapter 1), developmental regulation has so far been shown only for the oxidation pathway to 3-dehydro-20-HE.

#### Ecdysone oxidase

This enzyme irreversibly catalyses the conversion of ecdysteroids to 3-dehydro derivatives (Koolman and Karlson, 1975; 1978). Its presence has been demonstrated in several insect orders, including the Lepidoptera. The 3-dehydro ecdysteroid derivatives may be converted to hormonally less active 3-epi-ecdysteroids by ecdysone dehydrogenase isomerase (Nigg et al, 1974). Ecdysone oxidase has been shown to be developmentally regulated in Calliphora vicina (Koolman et al, 1978) where the activity rises late in the last larval instar and remains elevated during the pupal stage.

The pathway of metabolism of 20-HE may also

vary qualitatively. In the fleshfly, Sarcophaga peregrina, injected 20-HE was inactivated by conjugation to a glucolase sensitive polar substance during the last larval instar. In the puparium, however, epimerisation to 3-epi-20-HE appeared as a metabolic pathway undetected in the larval stage (Moribashi and Ohtaki, 1978). It was postulated that this new inactivating process functions to produce a rapid decrease in the titre of active 20-HE after pupation and may even be induced by 20-HE, suggesting a feedback control mechanism for 20-HE titre.

#### Status of haemolymph ecdysone

Ecdysone probably only transiently exists free in the haemolymph. Either it may be produced as a conjugate as suggested by Willig et al (1971) or rapidly bound by specific binding proteins in the haemolymph (Feyereisen, 1977). This kind of conjugation reaction may prevent metabolism and excretion of ecdysteroids, or may regulate the uptake of the hormone at the target tissue. In general, there is a lack of data describing the contribution of such reactions to

the regulation of hormone levels available at the tissues.

#### Ecdysteroid excretion

While it appears that ecdysteroids are generally converted to less active metabolites prior to excretion, the direct excretion of active hormone can occur (see Chapter 1). Koolman et al (1975) found that the rate of ecdysone excretion varies during locust development, with minimal rates coinciding with peak ecdysteroid titre and maximum excretion 24 hours later. It appears that the PG, or their secretory product, play a role in reducing excretion of ecdysteroids at the time when a high titre is normally present (Hoffman and Koolman, 1974). Ecdysteroid excretion also varies during Rhodnius development (Steele et al, 1982).

All these processes, and perhaps others yet to be discovered, directly influence the titre of 20-HE experienced by the tissues. The most information is available for Locusta where, whilst PG secretory activity is regulated (Hirn et al, 1979), it is likely that fluctuations in



other processes, outlined above, contribute much to the control of ecdysteroid titre.

In Manduca, much less information is available concerning regulation of the ecdysteroid metabolism. It is known that ecdysone 20-hydroxylase (= ecdysone monooxygenase) activity in Manduca hindgut is temporally regulated, being detectable only late in the fifth instar and early in the pupal instar (Nigg et al, 1976). It is likely that other aspects of ecdysteroid metabolism are also developmentally regulated in Manduca, acting to transform the relatively small changes in ecdysone production rates (see Fig. 4.8) to the dramatic fluctuations of ecdysteroid titres seen in the whole animal (Fig 4.9).

To summarise, numerous factors act to regulate the levels of active ecdysteroids at the target tissue. Whilst the rate of ecdysone production by the PG varies in a manner likely to contribute to the control of the titre of free hormone, variations in ecdysteroid metabolism probably account for the fine control required to produce the fluctuating titres essential to successful metamorphosis.

## APPENDIX A

Composition of Grace's tissue culture medium  
(Gibco).

<u>Inorganic salts</u>	mg/L
CaCl <sub>2</sub>	750
KCl	4100
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2280
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2780
NaHCO <sub>3</sub>	350
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	1013

### Other Components

Alpha-Ketoglutaric acid	330
Fructose	400
Fumaric acid	55
Glucose	700
Malic acid	670
Succinic acid	60
Sucrose	26680

## Amino acids

<b><math>\beta</math>-Alanine</b>	<b>220</b>
<b>L-Alanine</b>	<b>225</b>

<u>Amino acids (Continued)</u>	mg/L
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L-Arginine HCl	700
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L-Asparagine	350
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L-Aspartic acid	350
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L-Cystine	22
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L-Glutamic acid	600
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L-Glutamine	600
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Glycine	650
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L-Histidine	2500
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L-Isoleucine	50
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L-Leucine	75
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L-Lysine HCl	625
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L-Methionine	50
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L-Phenylalanine	150
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L-Proline	350
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DL-Serine	1100
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L-Threonine	175
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L-Tryptophan	100
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L-Tyrosine	50
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L-Valine	100
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<u>Vitamins</u>	
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Biotin	0.01
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D-Ca pantothenate	0.02
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Choline chloride	0.20
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<u>Vitamins (continued)</u>	mg/L
Folic acid	0.02
i-Inositol	0.02
Niacin	0.02
Para-aminobenzoic acid	0.02
Pyridoxine HCl	0.02
Riboflavin	0.02
Thiamine HCl	0.02

## APPENDIX B

### Composition of Manduca saline

#### Solution A:

	g/L
NaCl	0.259
KCl	3.31
MgCl <sub>2</sub> .6H <sub>2</sub> O	4.06
Sucrose	92.2
Tris	0.202

pH adjusted to 6.5 with 0.1M HCl.

#### Solution B:

	g/L
CaCl <sub>2</sub>	3.32

Solutions A and B were sterilised by autotoclaving and mixed in the ratio 9:1.

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